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CONJUGATES OF HUMAN MUCIN AND A CARBOHYDRATE POLYMER AND THEIR USE IN CANCER TREATMENT

Abstract:

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Conjugates between one or more repeated subunits of an antigen and a carbohydrate polymer are desired. Also described are immunogenic vaccines against disease states which contain the conjugates and methods for inducing cell-mediated immune responses. The conjugates may especially contain polymers of the carbohydrate mannose and one or more repeated subunits of human mucin.

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CONJUGATES OF HUMAN MUCIN AND A CARBOHYDRATE POLYMER AND THEIR USE IN CANCER TREATMENT

This invention relates to the immunotherapy of disease states, and in particular, but not exclusively to the immunotherapy of carcinomas.

Cancer is a major cause of death and severe trauma in modern society. Cancer is no respecter of persons as the young, old, males, females and peoples of all races may contract cancer, although cancer in children is relatively rare, perhaps with the exception of childhood leukemia. In western society, cancer of the colon and lung cancer are major diseases. In women, breast cancer is the most common form of cancer.

Many cancers are accompanied by overproduction of human mucin. Mucins are heavily glycosylated proteins (greater than about 100Kd) which are produced by many epithelial cells and tumours (1). Mucins found on cancer cells are different in some respects to those on normal epithelial cells, in that some mucins have a deficiency in their carbohydrate coat which leaves the protein core exposed. (2). There are seven forms of known human mucin designated MUC1, MUC2, MUC3, MUC4, MUC5 MUC6 and MUC7 (3, 4, 26, 27). MUC1 is the most ubiquitous. The various mucins all have very similar properties, that is, they are transmembrane glycoproteins, all having a variable number of repeated amino acid sequences, which have a high content of serine, threonine and proline. Overproduction of aberrantly glycosylated mucins (either non-glycosylated or a deficiency in glycosylation) is characteristic of tumours of the breast, ovary, pancreas, colon, lungs, prostate and other tumours of secretory tissue. The cDNA sequences of the respective protein cores of the human mucins MUC1 to MUC7 have been cloned and characterized and have been found to contain highly repetitive central portions of varying numbers of repeats of particular amino acid motifs (known as VNTR's). By way of example, MUC1 consists of unique amino and carboxyl terminal sequences separated by a highly repetitive central portion containing forty

to eighty tandemly arranged copies or repeats of a twenty amino acid motif. The VNTR's of MUC1 through MUC7 are set forth below:

MUC1 VNTR - SAPDTRPAPGSTAPPAHGVT
MUC2 VNTR - PTTTPISTTTMVTPTPTPTGTQT
MUC3 VNTR - HSTPSFTSSITTTETTS
MUC4 VNTR - TSSASTGHATPLPVTD
MUC5B VNTR - 87 base pair degenerate tandem repeats without peptide repeats
MUC5C VNTR - PTTSTTSA (494 base pair insert - eight amino acid tandem repeat)
MUC6 VNTR - 169aa (507 base pair) repeat unit
MUC7 VNTR - TTAAPPTPPATTPAPPSSSAPPE

The repeated subunit of MUC6 comprises 169 amino acids, although at this time the amino acid sequence of this repeat unit has not been fully characterized. The MUC7 sequence has recently been published (27).

Finn and colleagues have demonstrated that in the lymph nodes of patients with breast cancer (5, 6), cancer of the pancreas, ovary and other tumours, cytotoxic lymphocytes are present which react with human mucin. Antibodies to the MUC1 peptide can block the activity of these cytotoxic T-lymphocytes on MUC1$^+$ target cells (5, 6). Recently, cytotoxic lymphocytes to a murine lung cancer have also been described (28).

The surgery associated with tumour removal is traumatic to the patient, often disfiguring, and costly. Established chemotherapeutic and radiation procedures for tumour treatment which may be carried out in place of or in conjunction with surgical procedures are often debilitating and associated with severe side-effects. There is accordingly an urgent need for therapeutic compounds and methods for the prevention/treatment of tumours.

There is an urgent need for new compounds and methods for the treatment of cancer. Similarly, there is a pressing need for alternative compounds and therapies for the treatment

of other disease states such as type I allergies, malaria, HIV, dental caries, flu, cholera, foot and mouth disease, meningitis, Leishmania infection, whooping cough, rabies, Streptococcus infection, respiratory infection, measles, Lyme disease, tuberculosis, bacterial meningitis, shingles, rubella, hepatitis, herpes, hepatitis A, polio, venereal disease/trachoma, hepatitis B, common cold, cervical cancer, meningitis/pneumonitis, chicken pox, small pox, pneumonia/PUO.

In accordance with the first aspect of the present invention, there is provided a compound comprising a conjugate between an antigen and a carbohydrate polymer.

In accordance with another aspect of the present invention, there is provided a compound comprising a conjugate between the human mucin polypeptide, one or more repeated subunits thereof, or a fragment of said repeated subunits, with a carbohydrate polymer.

In a preferred embodiment of the present invention, the carbohydrate polymer is a polymer of the carbohydrate mannose.

Insofar as the present invention is concerned, the antigen can be a human autoantigen or a peptide antigen derived from a virus, microorganism or plant or an amino acid subunit of at least five amino acids in length of a human autoantigen or a peptide antigen derived from a virus, microorganism or plant. The antigen of the present invention can also consist of more than one, five or more amino acid subunits (as mentioned above) linked together. These linked subunits may be from the same or different origins within the bounds described above.

Examples of the antigens envisaged by the present invention are as follows: pollens, hepatitis C virus (HIV) core, E1, E2 and NS2 proteins, Plasmodium falciparum circumsporozoite protein, HIV-gp120/160 envelope glycoprotein, streptococcus surface protein Ag, influenza nucleoprotein, haemagglutinin-neuraminidase surface infection, TcpA pilin subunit, VP1 protein, LMCV nucleoprotein, Leishmania major surface glycoprotein (gp63), Bordetella pertussis surface protein, rabies virus G protein, Streptococcus M protein, Syncyticial virus

(RSV) F or G proteins, Epstein Barr virus (EBV) gp340 or nucleoantigen 3A, haemagglutinin, Borrelia burgdorferi outer surface protein (Osp) A, Mycobacterium tuberculosis 38kDa lipoprotein or Ag85, Neisseria meningitidis class 1 outer protein, Varicella zoster virus IE62 and gpI, Rubella virus capsid protein, Hepatitis B virus pre S1 ag, Herpes simplex virus type I glycoprotein G or gp D or CP27, Murray valley encephalitis virus E glycoprotein, Hepatitis A virus VP1, polio virus capsid protein VP1, VP2 and VP3, chlamydia trachomatis surface protein, Hepatitis B virus envelope Ag pre S2, Human rhinovirus (HRV) capsid, papillomavirus peptides from oncogene E6 and E7, Listeria surface protein, Varicella virus envelope protein, Vaccinia virus envelope protein, Brucella surface protein, a combination of one or more of said antigens, an amino acid subunit of said antigens comprising five or more amino acids in length or combinations of one or more of said subunits.

The antigens of the present invention can also consist of whole cells or sub-cellular fractions thereof. Such cells or sub-cellular fractions thereof may be derived from any tumour type or other source. Examples of cancer types from which the whole cells or sub-cellular fractions may be derived are breast, lung, pancreas and colon cancer and melanoma. Some further examples of specific antigens obtained from tumours are melanoma specific antigen (for example, the MAGE series antigen), carcino embryonic antigen (CEA) from colon and other cancers or indeed antigens extracted from any tumour.

This invention includes any one or more of the antigens listed and in particular includes any one or more of the human mucins MUC1 through MUC7 which, as mentioned above, all comprise highly repetitive central portions of repeated amino acid sequences which are high in serine, threonine and proline. In particular, the compounds of this invention may comprise a human mucin polypeptide (containing a variable number of repeats associated with normal allelic variation), or may comprise one or more of the repeated sequences of human mucin, preferably two to eighty, more preferably two to twenty and even more preferably two to ten repeated subunits of human mucin. The human mucin and subunits thereof are preferably non-glycosylated or aberrantly glycosylated so as to provoke an immune response to the

mucins found on cancer cells which have a deficiency in their carbohydrate coat which leaves the protein core exposed. The use of human mucin MUC1 is particularly preferred although it is to be clearly understood that the invention extends to the use of any antigen and especially to the use of the human mucins MUC1 through MUC7. For the purpose of convenience, the term MUC will hereafter be used to refer to any of the human mucins MUC1 through MUC7 and repeated subunits thereof. While only the human mucins will be dealt with hereafter, it must be kept in mind that his invention equally relates to any other antigen as mentioned previously.

Fragments of MUC may also be conjugated to a carbohydrate polymer. These fragments would generally comprise from five to twenty amino acids from the repeated amino acid sequences of any of mucins MUC1 through MUC7. For example, a fragment of the mucin MUC1 may comprise the amino acid sequence APDTR, APDTRPAPG, DTRPAPGSTAPP, and the like. For convenience of description these fragments are also included with the definition MUC. Similarly, other antigen fragments comprising at least five amino acids may be conjugated to a carbohydrate polymer.

A specified antigen (such as MUC1, MUC2, MUC3, MUC4, MUC5, MUC6 or MUC7) may form part of a fusion protein in order to facilitate expression and purification on production of the fusion protein in recombinant host cells. The non-antigen portion of the fusion protein would generally represent the N-terminal region of the fusion polypeptide with the carboxy terminal sequences comprising antigen sequences. Fusion proteins may be selected from glutathione-S-transferase, β-galactosidase, or any other protein or part thereof, particularly those which enable affinity purification utilizing the binding or other affinity characteristics of the protein to purify the resultant fusion protein. The protein may also be fused to the C-terminal or N-terminal of the carrier protein. The nature of the fusion protein will depend upon the vector system in which fusion proteins are produced. An example of a bacterial expression vector is pGEX which on subcloning on a gene of interest into this vector produces a fusion protein consisting of glutathione-S-transferase with the protein of interest. Examples of other vector systems which give rise to fusion proteins with a protein of interest

are described in Sambrook et al (7), which is incorporated herein in its entirety by reference. These can be included or cleaved; if included they could have a "carrier" function.

The protein or fusion protein maybe expressed in a number of prokaryotic (*E.coli* or *β-sutilis)* or eukaryotic *(baculovirus, CHO cells, cos cells or yeast)* expression systems. In some of these systems, for example, *baculovirus* or yeast, by introducing glycosylation motifs into the protein or fusion protein, the mannose rich glycosylation may be adequate; negating the need for chemically linking with mannose rich carbohydrate polymers. These novel fusion proteins may be used with or without mild periodate oxidation.

The carbohydrate portion of the compounds of the invention may comprise any carbohydrate polymer, for example, selected from polymers of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhamnose, 6-0-methyl-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose and α abequose, conformation and configuration isomers thereof, or a carbohydrate formed of two or more different monomer units. The number of repeated monomer units in the polymer is not important but generally carbohydrate polymers would comprise at least twenty monomer units, preferably in excess of one hundred monomer units, more preferably in excess of one thousand monomer units, and still more preferably in excess of ten thousand monomer units or more. Carbohydrate polymers may be a mixture of polysaccharide chains of varying molecular weights. Most preferably the carbohydrate polymer is a polymer of mannose or is a carbohydrate polymer containing mannose units.

Antigens may be conjugated to a carbohydrate polymer according to standard processes well known in the art of carbohydrate chemistry for the derivatization and reaction of polysaccharides and monosaccharides. Carbohydrates may be oxidized with conventional oxidizing reagents such as sodium periodate to give a polyaldehyde which is then directly reacted with the antigen (such as repeated subunits of MUC1) where amino functional groups on the protein chain (such as the ϵ group of lysine) react with the aldehyde groups which may

optionally be further reduced to form a Schiff base. Polysaccharide chains may be first activated with cyanogen bromide and the activated polysaccharide then reacted with a diamine, followed by conjugation to the antigen to form a conjugate which may optionally then be oxidized. The carbohydrate and polypeptide may be derivatized with bifunctional agents in order to cross-link the carbohydrate and polypeptide. Commonly used cross-linking agents include 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicyclic acid, homobifunctional imidoesters including disuccinimidyl esters such as 3,3'-dithiobis(succinimidyl-propionate), and bifunctional maleimides such as bis-N-maleimido-1, 8-octane. Derivatizing agents such as methyl-3-[(p-azido-phenyl)dithio] propioimidate yield photactivitable intermediates which are capable of forming cross-links in the presence of light. Oxidized carbohydrates may be reacted with hydrazine derivatives of antigens to give a conjugate. Alternatively, carbohydrates may be reacted with reagents such as carbonyl diimidazole, which after oxidation gives the desired conjugate.

The coupling of antigens to a carbohydrate involves converting any or all of the functional groups on the carbohydrate to reactive groups and thereafter reacting the reactive groups on the carbohydrate with reactive groups on the polypeptide. Carbohydrate polymers are replete with hydroxide groups, and in some instances, carboxyl groups (such as in idruionate), ester groups (such as methylgalacturonate) and the like. These groups may be activated according to standard chemical procedures. For example, hydroxyl groups may be reacted with hydrogen halides, such as hydrogen iodide, hydrogen bromide and hydrogen chloride to give the reactive halogenated polysaccharide. Hydroxy groups may be activated with phosphorous trihalides, active metals (such as sodium ethoxide, aluminium isopropoxide and potassium tert-butoxide), or esterified (with groups such as tosyl chloride or acetic acid) to form reactive groups which can be then be reacted with reactive groups on the polypeptide to form one or more bonds. Other functional groups on carbohydrates apart from hydroxyl groups may be activated to give reactive groups according to well known procedures in the art.

Polypeptides comprising MUC or other antigens may be produced according to well known procedures such as peptide synthesis, protein purification, or expression of polypeptides in host cells. Peptide synthesis may be employed for polypeptides containing up to about a hundred amino acids (for example, five repeated subunits of MUC1). Generally, for polypeptide containing about twenty or more amino acids, the preferred means of production is recombinant expression in a host cell, preferably a prokaryotic host cell, and more preferably a bacterial host cell. However, as discussed earlier, eukaryotic systems may also be used. Procedures for expression of recombinant proteins in host cells are well established, see, for example, Sambrook, et al (7).

Carbohydrates may be purified from natural sources or synthesized according to conventional procedures. Carbohydrates are available commercially from many suppliers.

In another aspect, the invention relates to an immunogenic vaccine against human disease states and in particular against tumour cells expressing human mucin or a subunit thereof, which comprises a compound comprising a conjugate between an antigen and a carbohydrate polymer, in association with a pharmaceutically acceptable carrier. Antigens which may be used in this aspect of the invention are as previously described. The vaccine is administered to human patients to protect against various disease states including cancer cell growth, and in particular, the growth of tumours of secretory tissues, such as tumours of the breast, colon, lung, pancreas, prostate, and the like. Patients may be immunized with the vaccine to protect against tumour formation of secretory tissues. Alternatively, patients suffering from tumours may be immunized with the vaccine as part of a therapeutic regimen for tumour treatment. By way of example, to protect women from breast cancer, women may be immunized with the vaccine pre- or post-puberty and may receive one or more injections, preferably an initial immunization, followed by one or more booster injections separated by several months to several years. In one immunization schedule, women may be immunized with the compounds of the invention and then receive a booster immunization at appropriate intervals. Further booster immunizations are then provided at regular intervals. The route of immunization is

no different from conventional human vaccine administration. Accordingly, vaccines may be administered subcutaneously, intramuscularly, orally, intravenously, and the like.

Some other disease states which may be protected against in this manner include, type I allergies, malaria, HIV, dental caries, flu, cholera, foot and mouth disease, meningitis, Leishmania infection, whooping cough, rabies, Streptococcus infection, respiratory infection, measles, Lyme disease, tuberculosis, bacterial meningitis, shingles, rubella, hepatitis, herpes, hepatitis A, polio, venereal disease/trachoma, hepatitis B, common cold, cervical cancer, meningitis/pneumonitis, chicken pox, small pox, pneumonia/PUO.

The amount of compounds of the invention or compositions thereof delivered to a patient is not critical or limiting. An effective amount of a compound of the invention is that which will stimulate an immune response against the antigen component. The amount of compounds or compositions delivered may vary according to the immune status of the patient (depending on whether the patient is immunosuppressed or immunostimulated), the judgement of attending physician or veterinarian whether the compound is used as a vaccine to prevent or treat a disease state or as a vaccine to prevent tumour formation, or whether the vaccine is used in the treatment of an existing tumour. By way of example, patients may receive from 1μg to 10,000μg of the compounds of the invention, more preferably 50μg to 5,000μg, still more preferably 100μg to 1,000μg, and even more preferably 100μg to 500μg of the compounds of the invention. Adjuvants are not generally required. However, adjuvants may be used for immunization. Suitable adjuvants include alum, as well as any other adjuvant or adjuvants well known in the vaccine art for administration to humans.

Compounds of the invention may be administered to patients in concert with a cytokine or other immune regulator. By way of example, immune regulators which may be administered in concert with the compounds of the invention include one or more of GM-CSF, G-CSF, M-CSF, TNFα or β, interferon α or γ, any of IL1 through IL13, or any other cytokine. The immune regulator may be administered at the same time as the compounds of the invention,

optionally as part of a multi-component administration form. Alternatively, the compounds of this invention and immune regulators may be administered at different time intervals.

In a still further aspect of this invention, there is provided a method for inducing a cell mediated immune response against antigens which comprises administering to an animal (including a human) a compound comprising a conjugate between said antigen and a carbohydrate polymer, optionally in association with a pharmaceutically acceptable carrier.

The immunization of humans and animals with the compounds of this invention may provoke a potentiated cellular response of activated T-lymphocytes which are cytotoxic to cells expressing the antigen component. By way of example, humans and animals may be immunized against tumours which express human mucins. A potential benefit of this invention arises from the fact that animals may be protected against cancer prior to tumour growth, as the compounds of the invention may provoke a cellular immune response of cytotoxic T-cells which kill tumour cells expressing mucin or other antigenic determinants. This invention is applicable to the immunization against tumours of secretory tissue, such as adenocarcinomas, more particularly, tumours of the breast, ovary, pancreas, colon, lung, prostate and the like.

The compounds of the invention may also be used as therapeutic agents for the treatment of patients suffering from cancer, as a part of the overall treatment for eradication of the cancer. Thus, the compounds of the invention may be administered to patients suffering from cancer either before or after surgery to remove the tumour. Preferably the compounds are administered as part of a chemotherapeutic regime following tumour excision. In these circumstances, the compounds of the invention are administered in amounts consonant with standard chemotherapeutic regimes for the administration of cytotoxic compounds for use in tumour treatment.

The compounds of this invention can also be used in immunization for therapy or prophylaxis of other disease states as mentioned earlier.

In a still further aspect, the invention relates to the use of a compound comprising a conjugate between the human mucin polypeptide, one or more repeated subunits thereof, or a fragment of said repeated subunits and a carbohydrate polymer in the treatment of adenocarcinoma, particularly breast cancer.

The compounds of this invention possess the advantage of being substantially non-toxic on administration to animals or humans, and as a consequence the compounds are well tolerated by administration to patients.

The invention described herein is not restricted to the human mucin MUC1. The invention clearly extends to the use of other mucins expressed by cancer cells, as well as to the use of other antigens which on coupling to polysaccharides, can be used to provoke cytotoxic T-cell responses against tumour cells, which compounds may be used in vaccines to prevent tumour formation, as well as for the treatment of cancer, and/or the treatment or prophylaxis of other disease states as mentioned earlier.

The invention will now be described with reference to the following non-limiting Examples.

The following abbreviations are used in the Examples:

ABBREVIATIONS

ELISA:	enzyme linked immunosorbent assay
DTH:	delayed type hypersensitivity
FP:	fusion protein
GST:	glutathione-S-transferase
HMFG:	human milk fat globule
Kd:	kilodalton
KLH:	keyhole-limpet haemocyanin
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline

SDS: sodium dodecyl sulphate

Tc: cytotoxic T-lymphocytes

VNTR: variable number of tandem repeats

CTL: cytotoxic T-cells

M-FP: mannan fusion protein

MHC: major histocompatability complex

MSA: mucin serum antigen

CASA: circulating MUC1 serum antigen

FIGURE LEGEND

Figure 1: Growth of 5 x 10^6 3T3 and MUC1$^+$3T3 cells is BALB/c mice.

Figure 2: Dose response of MUC1$^+$3T3 cells in (a) mannan-fusion protein and (b) non immunized BALB/c mice. Doses ranging from 10^6- 5 x 10^7 cells. Growth of 5 x 10^6 3T3 tumor cells is also shown.

Figure 3: Mice immunized with (a) mannan, mixture of mannan + fusion protein, M-FP and a control group (immunized with PBS); (b) 16.1FP-mannan, oxidized mannan, M-FP and PBS; (c) dextran-FP (D-FP), M-FP and PBS, and challenged with 10^6 MUC1$^+$3T3 cells.

Figure 4: BALB/c mice immunized with M-FP and treated with anti-CD3, anti-CD4 and anti-CD8 on -2, 0, + two days. Challenge with 10^6 MUC1$^+$3T3 cells.

Figure 5: DTH response measured at forty eight hours in mice immunized with mannan-fusion protein and challenged with dead (freeze-thaw 5 times) 3T3 and MUC1$^+$3T3 cells, Cp13-32-KLH, fusion protein, HMFG, mannan-fusion protein, GST, T4N1 and PBS in their hind footpads. Control (black box), mice treated with anti-CD4 (grey box) and mice treated with anti-CD8 (cross lines).

Figure 6: Cytotoxic T-lymphocyte assay with P815 and MUC1$^+$P815 ^{51}Cr labelled target cells.

Figure 7: A: (DBA/2^{++} x BALB/c)F1 mice were challenged with 5 x 10^6 MUC1$^+$P815 cells. After thirteen days of tumour challenge (established tumours) mice were immunized with 5μg M-FP (5μg corresponding to the amount of FP) once or every other day. Control mice were injected with PBS;
B: DBA/2^{++} mice were challenged with 5 x 10^6 MUC1$^+$P815 cells. After fifteen days of tumour challenge (established tumours) mice were immunized with 5μg M-FP (5μg corresponding to the amount of FP) once or every other day. Control mice were injected with PBS.

Figure 8: (A) CTL assay using ^{51}Cr MUC1-P815 and control P815 target cells BALB/c mice were immunized I.P. weekly x 3 and then spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector-to-target (E:T) ratios in 96 well U bottom plates and ^{51}Cr release was measured: percent specific release [(x - spontaneous release)/ (maximum release - spontaneous release)] x 100 is shown at various E:T ratios using spleens from mice immunized with MUC1$^+$ tumor (-▲-); MFP-oxidized (-●-); MFP-reduced (-■-); or FP (-+-) when tested on MUC1-P815 targets or on control P815 targets:- MUC1$^+$ tumor (-▲-); MFP-oxidized (-O-); MFP-reduced (-□-); or FP (-·-).
(B) Antibody test by ELISA: Sera from immunized mice (-▲- Tumor; -●- MFP-oxidised; -■- MFP-reduced; -□- FP; -O- saline (PBS)) were titrated on plates coated with 20μg/ml FP, washed and the bound antibody was detected with sheep anti-mouse globulin conjugated to horseradish peroxidase and developed as described (6); the absorbance at 405nm is recorded for various dilutions of antibody.
(C) DTH responses were induced in cyclophosphamide treated mice (200mg/kg) by IP immunization as above; six days later, footpads were

injected with 10^5 killed (freeze/thawed five times) MUC1-3T3 and 3T3; 50μg FP or (PBS) and the thickness of injected and control footpads was recorded at forty eight hours. The increase in footpad size is recorded ($cm^2 \pm$ standard deviation) in mice immunized with Tumor cells (-■-); MFP oxidised (-□-) or FP (-□-) and challenged as shown. There were five mice per group.

Figure 9: Growth of MUC1-3T3 tumors in five BALB/c mice per group after immunization with (-▲-) Tumor cells; (-●-) MFP-oxidised; (-■-) MFP reduced; (-□-) FP or (-O-) PBS, showing the size of the tumors (the product of two diameters), error bars, and the days after test tumor injection. (A) challenge with 10^6 MUC1-3T3 cells, (B) challenge with 5 x 10^6 MUC1-3T3 cells.

Figure 10: CTL assay using ^{51}Cr MUCI-P815(Tm211) ($H2^d$) and control P815 ($H2^d$) target cells. Various mice strains were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector:target ratios (E:T), and chromium release was measured and calculated as a percentage (a) NZB, B10D2, DBA2 and BALB/c are $H2^d$. (b) SJL $H2^s$, CBA $H2^k$, BALBb $H2^b$, BL6 $H2^b$, 129J $H2^b$, NZW $H2^z$.

Figure 11: CTL assay using (a) ^{51}Cr MUCI-P815 (Tm211) ($H2^d$) and control P815 ($H2^d$) target cells and E3 ($H2^b$) either peptide or not peptide pulsed BALB/c, BALBb, BL6 mice were immunized with M-FP and were used as effector cells. (b) ^{51}Cr BALB/cand BALB/b blasts, E3 and P815 MUC1 peptide pulsed. BALB/c mice were effectors. (c) ^{51}Cr BALB/c, and BALB/b blasts, E3 and P815 MUC1 peptide pulsed BALB/b mice were used as effectors. Spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector: target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 12: CTL assay using ^{51}Cr MUC1-P815 (Tm211) ($H2^d$) and control P815 ($H2^d$), 2R (K^kD^b), B6 (K^bD^b), CBA (K^k), 5R (K^bD^d) spleen cells were made as blast cells and peptide pulsed were also target cells B10A(2R) (K^kD^b) were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector:target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 13: CTL assay using ^{51}Cr MUC1-P815 (Tm211) ($H2^d$) and control P815 ($H2^d$). B6(K^bD^b), CBA (K^k), 5R (K^bD^d) spleen cells were made as blast cells and peptide pulsed were also target cells. B10A(5R)(K^bD^d were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector: target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 14: CTL assay using $^{51}CrP815(H2^d)$ and E3 ($H2^b$) target cells. bm-1 (K^bD^b), B6(K^bD^d), 5R (K^kD^b) and bm-12 (K^bD^d), spleen cells were made as blast cells and peptide pulsed were also target cells. B6(K^bD^b) were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector: target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 15: CTL assay using ^{51}Cr P815 ($H2^d$) and E3 ($H2^b$) target cells. bm-1 (K^kD^b), B6(K^bD^b), 5R(K^bD^d), and bm-12 (K^bD^d) spleen cells were made as blast cells and peptide pulsed were also target cells. bm-1 (K^bD^b) were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector:target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 16: CTL assay using ^{51}Cr P815($H2^d$) and E3 ($H2^b$) target cells. bm-1 (K^bD^b), B6(K^bD^d), 5R (K^bD^d) and bm-12 (K^bD^d), spleen cells were made as blast cells

and peptide pulsed were also target cells. bm-12 (K^bD^b) were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector:target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 17: CTL assay using ^{51}Cr P815 ($H2^d$) MUC1 peptide pulsed, and SjL ($H2^s$) and NZW ($H2^z$) spleen cells were made as blast cells and peptide pulsed were also target cells. SjL ($H2^s$) were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector:target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 18: CTL assay using ^{51}Cr P815 ($H2^d$) MUC1 peptide pulsed, and SjL ($H2^s$) and NZW ($H2^z$) spleen cells were made as blast cells and peptide pulsed were also target cells. NZW ($H2^z$) were immunized with MFP, spleen cells were collected and combined with ^{51}Cr labelled target cells at various effector:target ratios (E:T), and chromium release was measured and calculated as a percentage.

Figure 19: CTL assay using ^{51}Cr P815 ($H2^d$) MUC1 peptide pulsed cells. $\beta 2$-/ (K^bD^b), B6 (K^bD^b), 5R (K^bD^d), 2R (K^kD^b), bm-1 (K^bD^b) and NZW ($H2^z$) spleen cells were made as blast cells and peptide pulsed were also target cells. $\beta 2$-/- mice were immunized with MFP and their spleens were used as effector cells.

Figure 20: Mice immunized with different immunogens. Mice were bled and sera tested by ELISA for anti-MUC1 antibodies to (a) Fusion protein and (b) T4N1 (irrelevant peptide) coated plate. Absorbance values are at 1:100 antibody titer. Serum from mice immunized with T4N1 and the anti-MUC1 monoclonal antibody VA2 (isotope IgG1) were used as positive and negative controls.

EXAMPLE 1

Materials and Methods

Synthetic peptides, fusion protein, and HMFG production and immunization:

Peptides C-p13-32 (MUC1 VNTR), p31-55 and p51-70 (N-terminal to VNTR) and p344-364 and p408-423 (C-terminal to VNTR) were synthesized using an Applied Biosystems Model 430A automated peptide synthesizer (sequences shown in Table 1). The mouse CD4 N-terminal region peptide (T4N1) was also synthesized and used as a negative control peptide (Table 1). HMFG, was isolated from human milk (8). A fusion protein (9) containing 5 VNTR repeats was produced by subcloning the cDNA into the bacterial expression vector pGEX-3X (10) (Table 1).

BALB/c mice (females aged eight weeks) were immunized intraperitoneally with 50μg of either fusion protein, HMFG, C-p13-32 (coupled to diphtheria-toxoid or KLH (keyhole limpet hemocyanin) with glutaraldehyde) or T4N1 (coupled to diphtheria toxoid or KLH emulsified in complete Freund's adjuvant and this was repeated four and six weeks later in phosphate buffered saline. Prior to tumour injection and after tumour rejection mice were bled and the serum was tested on an ELISA for antibody production against the relevant immunogens.

MUC1$^+$3T3 tumour injections (see later description for production of these cells) were given subcutaneously in 0.2mls containing the appropriate tumour dose. Mice treated with anti-CD3, anti-CD4, anti-CD8 and anti-γ-interferon antibodies were given three intraperitoneal injections of 0.2mls on days -2, 0 and +2 (0 = day of tumour injection). Mice to be treated with antibody were injected subcutaneously with the tumour on day zero and on day five (tumour size approximately 0.15cm^2) when they were treated with rabbit complement (fresh serum - 0.2mls intravenously) and antibody (0.2mls intraperitoneally), on days five and seven.

TABLE 1: Sequences of synthetic peptides

Peptide	Amino Acid Sequence
MUC1 VNTR: Cp-13-32	C-PAHGVTSAPDTRPAPGSTAP
Fusion protein	(PAHGVTSAPDTRPAPGSTAP) x 5-GST
N-terminal region to MUC1: p31-55 p51-70	 TGSGHASSTPGGEKETSATQRSS VP RSSVPSSTEKNAVSMTSSVL
C-terminal to MUC1: p334-364 p408-423	 NSSLEDPSTDVVQELQRDISE TGFNQYKTEAASRVNL
Mouse CD4: T4N1	KTLVLGKEQESAELPCEY

Treatment of mice with antibodies:

To ensure that the antibodies to CD3, CD4 and CD8 were depleting or blocking $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, a serological analysis of residual cells was performed using the antibodies to CD3, CD4 and CD8. Spleen and lymph node cells were removed from normal and treated BALB/c mice, the lymphocytes were teased, washed in DME and incubated at 37°C for five minutes in 0.83% ammonium chloride to lyse red blood cells. Serology tests were performed where 2×10^5 spleen/lymph node cells from mice were added to a 1:500 dilution of anti-CD3, anti-CD4 and anti-CD8 ascites. Following extensive washing, the cells were incubated with (mouse thymus cell absorbed) rat anti-mouse IgG and incubated for thirty minutes on ice. Mice which had been treated with anti-CD3, anti-CD4, anti-CD8 or anti-CD4+CD8 were each tested with these antibodies. It was found that the $CD3^+$ cells were depleted and $CD4^+$ and $CD8^+$ cells had been blocked.

Preparation of soluble GST-MUC1 fusion protein

A 309 base pair insert (PDF9.3) encoding a little more than 5 repeats of a 60 base pair motif from the VNTR region of MUC1 cDNA (10) was subcloned into the bacterial expression vector pGEX-3X, in the correct reading frame and orientation (11). Fusion protein (FP), consisting of glutathione-S-transferase (GST, 26Kd) and MUC1 VNTR (12Kd), was induced with 0.1mM IPTG (11). Cells were collected by centrifugation, washed and lysed by

sonication in buffer containing 1% (v/v) Triton X-100. Supernatant containing the soluble FP was mixed with glutathione-agarose beads (sulphur-linked) (Sigma, St. Louis) and collected by centrifugation. The FP ((C-PAHGVTSAPDTRPAPGSTAP) x 5-GST) was eluted with buffer containing 5mM reduced glutathione, dialyzed against phosphate buffer and analyzed by SDS-PAGE.

Polyacrylamide gel electrophoresis

Samples to be tested were mixed with SDS sample buffer, boiled for five minutes and then loaded onto a 12.5% SDS-PAGE gel. Gels were stained in 0.2% Coomasie blue and then destained in 7% acetic acid or were silver stained (16). Molecular weight markers used: 200,000 myosin; 116,000 β-galactosidase; 92,500 phosphorylase *b:* 66,200 bovine serum albumin; 43,000 hen egg white ovalbumin; 31,000 bovine carbonic anhydrase; 21,500, soybean trypsin inhibitor, and 14,400 hen egg white lysozyme.

Conjugation of Mannan to MUC1 fusion protein

Mannan was oxidized to a poly-aldehyde by treating 14mg of mannan (a mannose containing polysaccharide) in 1ml of 0.1M phosphate buffer pH6.0 with 100μl 0.1M sodium periodate in phosphate buffer for 30-60 minutes at 4°C. Following a further thirty minute incubation at 4°C with 10μl ethandiol, the mixture was passed through a PD-10 column equilibrated in buffer pH6.0-pH9.0 and the mannan fraction collected; 900μg of MUC1 FP was added to the oxidized mannan, reacted overnight at room temperature and used for subsequent studies.

The fusion protein was radiolabelled with 125_I using chloramine-T. The unlabelled fusion protein was mixed with radiolabelled fusion protein such that the specific activity was 1 x 10^7cpm/μg and reacted with oxidized mannan as above. The mannan-FP was stabilized by reducing the Schiffs bases and residual aldehyde groups. The complex was then analyzed by SDS-PAGE, Autoradiography, coomasie (protein) stain, silver (protein +CHO) stain, and silver PAS (CHO) stain and by gel permeation chromatography using sepharyl S 208 column (1.5 cm x 100 cm).

Immunization schedule

BALB/c mice (females aged eight weeks) were immunized intraperitoneally with 5μg (corresponding to amount of FP) mannan-FP, FP and a mixture of non-conjugated mannan + FP in phosphate buffered saline (PBS) once weekly for three weeks. Mice were previously immunized with FP alone and this was used for a control for antibody production (see below). Prior to tumour injection, mice were bled and the serum tested by ELISA (see below) for antibody production against FP, (anti-mannan antibodies).

Tumours and antibodies

The BALB/c mouse fibroblast cell line 3T3 transfected with the MUC1 cDNA transmembrane form with the *ras* gene and a cell line MUC1$^+$3T3 was developed (obtained from Dr D Wreschner, Tel Aviv University, Israel). Mice received a 0.2ml subcutaneous injection of appropriate tumour cell dose in PBS and subsequent tumour growth measured. All measurements were performed with dial gauge callipers (Schnelltaster, H C Kroplin, Hessen, Germany) and the size of the tumours were expressed by the area of the tumour size (cm^2) (diameter x diameter). The murine DBA/2 mastocytoma cell lines P815, and MUC1$^+$P815 (containing the cDNA of the membrane anchored form of MUC1) were obtained from Dr B Acres (Transgene, Strasbourg, France).

Rat Mabs to murine CD3 (KT3.2), CD4 (H129.19) and CD8 (53-6.72) were prepared from ascites and tissue culture supernatants (12 to 14). Ascites fluid were prepared in SCID mice as described previously (15). Mice treated with anti-CD3, anti-CD4 and anti-CD8 antibodies were given three intraperitoneal injections of 0.2mls on days -2, 0 and +2 (0=day of tumour injection). MUC1 antibodies used were VA1 and VA2, produced against a GST-MUC1 bacterial FP which contains five VNTR repeats (16).

Preparation of peptides and HMFG

Peptides C-p13-32 (C-PAHGVTSAPDTRPAPGSTAP) (MUC1 VNTR) and T4N1 (KTLVLGKEQESAELPCEY) (mouse CD4 N-terminal region peptide) were synthesized using

an Applied Biosystems Model 430A automated peptide synthesizer. HMFG was isolated from human milk and prepared as previously described (17).

Enzyme linked immunosorbent assay (ELISA)

(a) Measurement of anti-fusion protein antibody: The ELISA test was performed (17), where 20μg/ml of FP was coated in the wells of a microtitre plate, non-specific binding blocked with 2% bovine serum albumin, and 50μl of serum from FP and mannan-FP immunized mice added for two hours at room temperature. Normal mouse serum (NMS) was used as negative control. After washing, sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase conjugate (Amersham, United Kingdom) was added, incubated at room temperature and the plate was developed using 50μl, 0.03% 2,2'-azino-di(3-ethylbenzthiazoline sulphonate (Amersham, United Kingdom), 0.02% H_2O_2 (100 Volume, Ajax Chemical) in 0.1M citrate buffer, pH4.0 and incubated for ten to fifteen minutes at room temperature until the desired intensity was achieved. Absorbency was read at 405nm in a plate reader.

(b) Determination of the activity of fusion protein after conjugation to mannan: The ELISA test was performed as described above with the following modifications; 20μg/ml of FP, mannan-FP and mannan were coated on the plate and the primary antibodies used were VA1 and VA2 (anti-FP Mabs).

Induction of DTH

To induce DTH in mice, cyclophosphamide (Endoxan-Asta, Mead Johnston) at a dosage of 200mg/kg body weight, was injected into the peritoneal cavity two days before an intraperitoneal injection of 5μg mannan-FP. Six days later, the hind footpads were injected (20μl) with either $10^5$3T3 or MUC1$^+$3T3 (freeze/thawed five times), 50μg of HMFG, FP, C-p13-32 (coupled to keyhole-limpet haemocynin using glutaraldehyde), T4N1 (an irrelevant peptide), mannan-FP, GST and mannan and an equivalent volume of PBS. The DTH response was measured at forty eight hours later, by measuring the width and the thickness

of the footpad and calculating their product. All measurements of footpads were performed with dial gauge callipers (Schnelltaster, H C Kroplin, Hessen, Germany).

Cytotoxic T-lymphocyte assay

BALB/c mice immunized with mannan-FP were sacrificed and their spleen cells were collected and washed in 2% foetal calf serum/PBS. The target cells, P815 and MUC1$^+$P815 cells were either not treated or treated with 5mM phenyl N-acetyl-a-D-galactosaminide (pagal) for two days (to inhibit O-linked glycosylation) (Sigma, St Louis, USA) prior to use in a standard ^{51}Cr release assay. Tumour cells (10^6 cells) (target cells) were radiolabelled with 100μCi of $Na_2{}^{51}CrO4$ (Amersham Corp, Arlington Heights) for sixty minutes at 37°C, followed by extensive washing. Spleen cells and target cells, were resuspended in culture medium, and then combined at various effector-to-target ratios in 96-well, U-bottom plates (Costar Corporation). The plates were then centrifuged at 100xg for three minutes to initiate cell contact and incubated for four hours at 37°C in 10% CO2. After incubation the supernatants were collected and radioactivity was quantitated in a gamma counter (Beckman Instruments).

Spontaneous release of ^{51}Cr was determined by incubation of the target cells alone, while maximum release of ^{51}Cr was determined by treatment with 10% sodium-dodecyl sulphate and percentage of specific release was determined as [(experimental - spontaneous)/(maximum - spontaneous)] x 100%.

T Proliferation Assay

Mice immunized with M-FP were sacrificed, their spleen cells were collected, washed in 2% foetal calf serum/PBS, red blood cells lysed with 0.14% NH_4Cl and duplicate cultures of 5 x 10^5 spleen cells in 100μl of culture media were seeded in a 96-microwell plate. Spleen cells were stimulated with 100μl of the following: 10μg - T4N1, GST, mannan, HMFG, Cp13-32, FP, MFP; and 10^5 breast cancer cells (pagal treated and untreated) of - 3T3, MUC1$^+$3T3, P815, MUC1$^+$P815, and 10^5 human breast cancer cell lines - T47D, MCF7 and ZR75. All tumour cells were treated with 25μg/ml of mitomycin-C (Sigma, Victoria Australia) for two

hours at 37°C to inhibit proliferation of the tumour cells. Cultures were incubated at 37°C in 5% CO_2 for thirty six hours. ^{3}[H]TdR (Amersham, United Kingdom) (6.7 Ci/mmol) incorporation was determined during the last four hours of culture (1μCi/well).

EXAMPLE 2

Serological analysis of MUC1$^+$3T3 cells:

In vitro MUC1$^+$3T3 cells did not appear to be different to normal 3T3 cells as they had the same appearance and growth characteristics. By serological analysis, MUC1$^+$3T3 cells expressed high concentrations of MUC1 and were H-2^{d+}. Antibodies to MUC1 VNTR peptides reacted with MUC1$^+$3T3 and MUC1$^+$P815 similarly to the human breast cancer cell lines T47D and MCF7 (typing with anti-HMFG: BC2 antibody, anti-fusion protein: VA1 and VA2 antibodies, and anti-MUC1 peptide antibodies: BCP7, BCP8, BCP9 and BCP10). However the murine tumour was differently glycosylated than the human tumour as MUC1$^+$3T3 and MUC1$^+$P815 cells were reactive with anti-carbohydrate (3E1.2) antibody (epitope: glycolylsialyl-Tn) but not with other antibodies to carbohydrate (CC5 -epitope: blood group Le^a). This shows that the protein antigens are intact, but the glycosylation is altered. This is not surprising as mice and humans have different glycosyl transferases and therefore different patterns of glycosylation. However, after removal of sugars by pagal treatment, the antibodies to MUC1 VNTR (non-APDTR reacting antibodies) which previously had weak or no reaction with cell lines, became reactive as their epitope has now exposed. There was no difference noted with the (AP)DTR(PA) reactive antibodies. There was a major difference in reactivity with the carbohydrate reactive antibody (3E1.2) where the staining became weak or negative after pagal treatment, indicating that the pagal was indeed removing O-linked sugars as the epitope of 3E1.2 is O-linked to the protein core of the mucin (18). The typing was repeated at different times and the same results were obtained, which indicated that the phenotype was stable.

In vivo growth of MUC1$^+$3T3 cells:

BALB/c mice received a subcutaneous injection of 5 x 10^6 MUC1$^+$3T3 or 3T3 cells and the subsequent growth measured; 3T3 cells grew progressively and were not rejected, as would

be expected in BALB/c mice. By contrast the MUC1$^+$3T3 cells grew progressively until day 10 when they started to shrink and had gradually disappeared by day eighteen. Thus, the human MUC1$^+$ gene product appears to confer an immunogenicity on 3T3 cells, leading to their rejection. This was indeed the case as the subsequent challenge with 5 x 10^6 MUC1$^+$ 3T3 or 3T3 cells demonstrated the total resistance in immunized mice to the growth of MUC1$^+$ 3T3 cells, whereas 3T3 cells grew - that is, the immunogenicity was found only in MUC1$^+$ bearing tumours and was specific for this antigen. Specificity and memory indicate an immune response to MUC1$^+$ and not some other effects such as MUC1$^+$ effecting the growth properties of 3T3. After several weeks of repeated experiments using tumours passaged *in vivo*, we noted that not all of the mice rejected their tumours and up to 30% of MUC1$^+$ tumours continued to grow. When these tumours were excised and MUC1$^+$ measured serologically, a proportion of cells in the tumours were MUC1$^-$, that is, some of the MUC1$^+$ transfected cells had lost their capacity to express MUC1$^+$ *in vivo* (we did not determine whether the genes were still present). Such observations have been reported elsewhere with rat tumours (19), presumably due to unstable expression of MUC1. In all our future studies we ensured that tumours were 100% MUC1$^+$ when used, by serologically testing the MUC1 expression with the anti-HMFG antibody BC2.

T-cell immune responses to MUC1$^+$3T3 cells:

Cellular immunity was most likely to be the mode of rejection as it is the commonest mode of rejecting tumour allografts in mice. This was confirmed by the ability of anti-CD3 antibodies to totally abrogate immunity. To determine whether CD4$^+$ or CD8$^+$ cells were responsible for rejection, mice received multiple doses of anti-CD4 or anti-CD8 antibody as these were known to cause immunosuppression in other models (20, 21). Functional CD4 cell depletion by blocking had a transient effect on tumour growth, and tumours were rejected in a similar fashion to untreated mice. By contrast, anti-CD8 treatment led to prolonged tumour growth. We conclude that CD3$^+$ cells are totally responsible for rejection, CD4$^+$ cells have a minimal effect and CD8$^+$ cells are the major effectors of graft rejection. It was noted that in anti-CD8 treated mice, the tumours were smaller than those receiving anti-CD3 - clearly the anti-CD8 antibody was not as effective as total T-cell removal with anti-CD3

antibody. CD4 cells having a minor effect was unlikely as the combined action of anti-CD4 and anti-CD8 was no better than anti-CD8 alone. However, we noted that anti-γ-interferon (γIFN) treatment (of no effect used alone) combined with anti-CD8 gave a similar effect with anti-CD3; thus γIFN plays a role in tumour graft rejection, which is mediated by $CD8^+$ cells and γIFN.

Thus, $MUC1^+$3T3 cells could immunize BALB/c mice against MUC1 carried on the 3T3 cells and gave rise to cellular immunity expressed by $CD3^+8^+$ cells but not by $CD3^+4^+$ cells; there was little humoral immunity as no anti-MUC1 antibodies were found. To measure the various parameters of the immune response, we examined (a) delayed type hypersensitivity and (b) cytotoxic T-lymphocytes.

(a) Delayed type hypersensitivity: Clearly the immune response was cellular and due to $CD8^+$ cells. To determine whether this also involved a DTH response (usually considered to be mediated by $CD4^+$ cells) or a cytotoxic T-cell response (usually $CD8^+$), mice were immunized with $MUC1^+$3T3 cells and a DTH was performed by injecting the hind footpads with various antigens. Preliminary studies demonstrated that in the absence of cyclophosphamide no measurable DTH responses occurred. A DTH response was detected in the footpads injected with killed (freeze/thawed five times) $MUC1^+$3T3 cells and a weaker response when challenged with either HMFG, fusion protein-GST and Cp13-32-KLH. These responses were clearly specific as 3T3 cells elicited no response. To determine whether the DTH response was mediated by $CD4^+$ or $CD8^+$ cells, mice were injected with anti-CD4 and anti-CD8 antibodies and the DTH response measured. Anti-CD4 totally blocked DTH reactions, anti-CD8 partially blocked DTH reactions, but to a lesser extent, when challenged with MUC1+3T3 cells, Cp13-32, HMFG and fusion protein. Thus the cells which cause the effective immune response to human MUC1 (CD8) were not the same as those eliciting a DTH response, although $CD8^+$ cells certainly contributed to the DTH.

(b) Cytotoxic T-lymphocytes: Cytotoxic assays were performed and after MUC1$^+$3T3 cell immunization there was up to 60% lysis of MUC1$^+$P815 targets treated with pagal. Untreated MUC1$^+$P815 targets and non-transfected P815 targets were not lysed. Pagal treated and non-treated 3T3 and MUC1$^+$3T3 targets also gave no lysis - possibly as 3T3 cells are poor targets for Tc assays. To determine the phenotype of the Tc, anti-CD4 and anti-CD8 antibodies were used in blocking studies - the anti-CD8 reagent (53-6.7) was known to be capable of blocking T-cell lysis by CD8$^+$ cells. This proved to be the case in these studies as anti-CD8 could block Tc, whereas anti-CD4 and a control antibody had little effect. Since only Tc were found to pagal treated MUC1$^+$P815 targets, and since non-APDTR reactive anti-MUC1 antibodies (VA1, BCP7, BCP9 and BCP10) became reactive with pagal treated MUC1$^+$3T3, MUC1$^+$P815, T47D and MCF7 cells, it is clear that both the antibody reactive and T-cell reactive epitopes are hidden, and both exposed after pagal treatment.

Mice resistant to MUC1$^+$3T3 cells have CD8$^+$ T-cell immunity, CD4$^+$ DTH, a detectable Tc response due to CD8$^+$ cells, and no antibody (see below). As the Tc response (at least at the level of the T-cell phenotype) correlated with the effector cell phenotype in rejecting tumours, it would appear to be the more appropriate response to measure.

Immune responses - B cells:

While it was shown above that cellular immunity was effective and little antibody was made, the role of antibody was not clear. Further, mice generally make poor antibodies and mobilize complement so poorly that they are not the species of choice on which to study antibody mediated destruction of grafts, unless certain conditions are met - a) the provision of sufficient antibody (be it polyclonal or monoclonal); b) the provision of sufficient complement; c) high density of surface antigens. The MUC1$^+$ antigen density is high so additional antibody and complement were provided. In addition, the mice were immunosuppressed with CD3 to remove any component of cellular immunity (22). In spite

of large amounts of antibody and complement (as described in materials and methods) (capable of rejecting skin allograft and xenografts), the tumours grew progressively - indeed, at the same rate as in mice not receiving antibody. Thus, antibody and complement are unable to cause rejection of MUC1$^+$3T3 cells.

Immunization with HMFG, peptides and fusion protein:

The preceding defines a model of the murine immune response to human MUC1 transfected into 3T3 cells and forms the basis for using various immunogens to generate a similar or greater immune response with synthetic materials as that produced with cellular MUC1. The aim was clearly to substantially decrease tumour growth after immunization. As immunogens, natural mucin (HMFG), synthetic products - MUC1 peptides made of VNTR dimers, and a 5 x VNTR repeat fusion protein were used. It should be noted that without prior immunization, tumours are rejected after eighteen days - such mice then being resistant to a subsequent challenge. Thus there is a "window" of approximately eighteen days when tumours will be rejected. So that, immunization could lead either to no tumours appearing or to reduced size during this time.

To examine the immunogenicity of HMFG, fusion protein and synthetic peptides groups of fifteen 15 BALB/c mice were immunized with 50μg of these materials and challenged with 1 - 5 x 10^6 3T3 or MUC1$^+$3T3 cells. The 3T3 cells had the same progressive growth in all immunized and non-immunized mice, so there were no non-specific effects of the immunization procedures. When mice were challenged with the lower dose of 1 x 10^6 cells, significant differences were noted as compared to the non-immunized control. Thus, on day six, mice immunized with either the peptide or fusion protein had tumours approximately 25% that of controls; immunizing with HMFG was less effective, tumours being approximately 60% the size of controls. However when challenged with 5 x 10^6 MUC1$^+$3T3 cells, there was some difference in tumour size, compared to the controls, but not as obvious by challenging with a lower dose. As expected with subsequent tumour challenge, the peptide immunized mice which had rejected the tumour were now resistant to tumour challenge. Thus, immunizing mice with peptides, fusion proteins or HMFG and challenging with a low

dose of MUC1$^+$3T3 cells gave rise to some anti-tumour effect. Although the VNTR containing peptide, fusion protein and HMFG gave some degree of protection, mice immunized with the N- and C-terminal peptides of MUC1 had no significant protection indicating that these peptides do not induce immunity to MUC1, and also showing that the immunization procedure itself was without effect. To measure the various parameters of the immune response, we examined (a) MUC1 antibody production, (b) delayed type hypersensitivity and (c) cytotoxic T-lymphocytes.

(a) Antibody: Immunized mice with peptides, fusion protein or HMFG had high levels of anti-MUC1 antibody both before and after tumour injection. Thus, immunization gave rise to high levels of antibody, but apparently little cellular immunity as shown by a minor effect on the tumours. It was of interest that mice immunized with the control peptide (T4N1), and which had rejected the tumour did not produce antibodies against MUC1; nor did the mice immunized with peptide and other immunogens have an increase in antibody titre after rejecting the tumour.

(b) DTH: Mice immunized with HMFG, Cp13-32 and fusion protein-GST had DTH responses to the various MUC1 antigens and which could be inhibited by CD4 (totally) and CD8 (partially) antibodies. Thus, immunization with the three agents gave rise to some degree of cellular immunity but not sufficient to greatly inhibit tumour growth.

(c) Cytotoxic T-lymphocyte assay: Tc assays were performed from spleen and lymph node cells of immunized mice and no cytotoxic cells were detected. Thus the various immunization procedures appeared to bias the immune response to antibody production, rather than cellular immunity.

Table 2 summarizes the differences in immunizing with cellular and synthetic antigens.

TABLE 2: Differences in immunizing with cellular and synthetic antigens

Immunogens	Tumour rejection	Antibody	DTH	Tc
Tumour MUC1$^+$3T3	+++	+	+++	+++
Peptide	+	+++	+++	-
Fusion protein	+	+++	+++	-
HMFG (mucin)	+	+++	+++	-

+++ = high; + = low; - = absent

EXAMPLE 3

Analysis of M-FP

The MUC1 FP was bound to mannan using periodate as described in the materials and methods. The amino groups of the FP reacts with aldehyde residues of the oxidized mannan to form the labile Schiff base (Figure 21). Free mannan and FP was not separated from conjugated mannan. Elution profiles for ^{125}I-FP and ^{125}I-M-FP obtained by gel filtration chromatography demonstrated that the mannan-fusion protein eluted as two peaks (201Kd and 73Kd). The FP eluted as two peaks: 38Kd and 20Kd (this lower peak may be GST due to cleavage of FP). Autoradiography analysis of ^{125}I-FP and ^{125}I-M-FP showed that most of the FP has been conjugated to mannan. Gel staining analysis of coommassie (protein), silver PAS) (carbohydrate) and silver (carbohydrate + protein) showed that M-FP conjugate runs as a smear and that it is a heterogeneous molecule. It showed that the coupling efficiency of FP to mannan was almost 100% (not shown)

The activity of FP after conjugation to mannan determined by an ELISA test showed that the FP had retained all its activity.

In vivo growth of MUC1$^+$3T3 cells

BALB/c mice which received a subcutaneous injection of 5 x 10^6 MUC1$^+$3T3 cells grew progressively until day ten when they started to shrink and disappeared by day eighteen, whereas 3T3 cells were not rejected as expected by BALB/c mice as set out in Example 2

(Figure 1). Thus, the human MUC1$^+$ gene product confers an immunogenicity on 3T3 cells, leading to their rejection, and such mice were totally resistant to subsequent challenge. Cellular immunity was the mode of rejection as anti-CD3 and anti-CD-8 antibodies totally abrogated immunity.

Immunization with Mannan-Fusion Protein

To examine the immunogenicity of the mannan-FP, groups of ten BALB/c mice were immunized with 5μg mannan-FP (5μg corresponding to the amount of FP) and challenged with 10^6 - 5 x 10^7 MUC1$^+$3T3 cells. There was no obvious tumour growth in immunized mice (Figure 2A) as compared to non immunized mice (Figure 2B). To show that the mannan-FP conjugate gave rise to specific anti-tumour immunity and that mannan or FP alone were without effect, mice were immunized with an equivalent dose of mannan (as in the conjugate = 7mg/ml), a mixture of mannan and FP and a group of non immunized mice and were challenged with 10^6 MUC1$^+$3T3 cells. Mice immunized with mannan-FP conjugate, no tumour growth was observed whereas mice immunized with mannan alone and a mixture of mannan and FP, tumours grew no different as compared to non immunized mice (Figures 3A, 3B and 3C). Thus, protection of tumour growth was specific for the conjugate and mannan and FP alone was without effect. Mannan-FP immunized mice were challenged with 10^6 3T3 cells and the 3T3 cells had the same progressive growth in immunized and non-immunized mice (Figure 3), indicating that there were no non-specific effects of the immunization procedures.

Immune response to M-FP

Anti-CD3 antibody could totally abrogate immunity in mice immunized with M-FP (Figure 4) and mice which received anti-CD4 or anti-CD8 antibodies showed the following effect: CD4 immunosuppression had a minor effect on tumour growth (Figure 4); by contrast anti-CD8 treatment led to prolonged tumour growth. Thus CD3$^+$/CD8$^+$ cells are totally responsible for the immunity and tumour protection, CD4$^+$ cells had a minimal effect (Figure 4). Thus M-FP could immunize against MUC1 carried on the 3T3 cells giving rise to cellular immunity expressed by CD3$^+$/CD8$^+$ cells but not by CD3$^+$/CD4$^+$ cells. To measure

the various parameters of the immune response, we examined (a) delayed type hypersensitivity, (b) cytotoxic T-lymphocytes (c) T-cell proliferation and (d) antibody production.

(a) *Delayed type hypersensitivity:* DTH responses (usually considered to be mediated by $CD4^+$ cells) were performed by injecting the hind footpads with the various antigens (Figure 5). A DTH response was detected in the footpads challenged with killed (freeze/thawed five times) $MUC1^+$3T3 cells, HMFG, FP-GST, Cp13-32-KLH, mannan-FP and a weaker response to GST (as GST is part of the FP) (Figure 5). These responses were clearly specific as killed 3T3 cells, mannan alone, an irrelevant peptide (T4N1) and PBS could elicit no responses. To determine whether the DTH response was mediated by $CD4^+$ or $CD8^+$ cells, mice were injected with anti-CD4 and anti-CD8 antibodies and the DTH response measured. Anti-CD4 totally inhibited DTH reactions, anti-CD8 inhibited but to a lesser extent (Figure 5). Thus the cells which caused the effective immune response to human MUC1 ($CD8^+$ cells as shown in Figure 4) were not the same as those eliciting a DTH response, although $CD8^+$ cells certainly contributed to the DTH.

(b) *Cytotoxic T-lymphocytes:* Cytotoxic assays were performed and it was shown that after M-FP immunization there was 50% MUC1 specific lysis of $MUC1^+$P815 targets (Figure 6) down to ratio of 12:1. Non-transfected P815 targets were not lysed (Figure 6). It is likely that these cells were $CD8^+$ (12).

(c) *T cell proliferation:* Proliferation assays were performed and it was shown that after M-FP immunization there were proliferative T-cells to M-FP, FP, Cp13-32, HMFG, and to pagal treated and untreated $MUC1^+$3T3, $MUC1^+$P815 cells. Other stimulants had no effect. In another assay different peptides were added at a range of peptide doses (1μg-80μg). A dose response was observed.

cp13-32, Cp-24, p13-32, p1-24, Ap1-15 and p5-20 were positive of the peptides p5-20, p14-24 and p16-24, p5-20 was positve but p14-24 and p16-24 were negative. It seems that the epitope is not likely to be the antibody epitope APOTR, but is in p14-24 - possibly GSTAP. the epitope of the T cell is further being investigated.

The sequence numbering is as follows:

1 5 10 15 20 21 next repeat

P D T R P A P G S T A P P A H G V T S A P ----------

(d) *Antibodies to Mannan GST-MUC1 fusion protein conjugate:* Mice were bled and their sera tested by ELISA for anti-FP antibodies. No anti-FP antibodies were detected compared to mice immunized with FP alone. Plates coated with mannan coupled to BSA were used to detect anti-mannan antibodies and no anti-mannan antibodies were detected. Normal mouse serum was as a negative control.

Thus, mice made totally resistant to MUC1$^+$3T3 cells by immunizing with FP coupled to mannan have CD8$^+$ T-cell immunity, CD4$^+$/CD8$^\pm$ DTH, a detectable Tc response due to CD8$^+$ cells, proliferative T-cells to the specific to the immunizing antigen, and little humoral immunity as no anti-MUC1 antibodies were found M-FP appears to be able to induce an anti-tumour response, similar to that shown with tumour cell rejection (12).

M-FP in therapy

To determine the effectiveness of M-FP as a therapeutic agent against established tumours, injections of MFP were delayed until tumours were established. MUC1$^+$P815 cells grow progressively in (DBA/2 x BALB/c)F1 mice, when given subcutaneously, until day sixteen to twenty when they start to shrink and usually disappear by day twenty-eight (Figure 7A). To examine the effectiveness of M-FP on established tumours, groups of five (DBA/2 x BALB/c)F1 mice were injected with 5 x 10^6 MUC1$^+$P815 cells; thirteen days later, the mice were injected with 5μg M-FP (5μg corresponding to the amount of FP) once, or every other day. Tumours in control mice (injected with PBS) were rejected by day twenty-eight.

However, mice injected regularly began to reject their tumours immediately, the tumours rapidly disappearing by day twenty. A single injection also gave rise to more rapid rejection (Figure 7A).

Another model was examined using MUC1$^+$P815 cells in DBA/2 mice, which grow until day twenty-two to thirty when they shrink and usually disappear by day forty two. MUC1$^+$P815 cells were injected subcutaneously and on day fifteen DBA/2 mice were injected with MFP. In the control group mice injected with PBS, tumours were rejected by day forty-two whereas mice immunized with MFP once on day fifteen tumours were rejected by day thirty-three (Figure 7B) compared with mice immunized every other day, tumours were rejected by day twenty-seven with a rapid reversal in their growth after a single injection. Thus, not only do pre-immunized mice have an anti-tumour effect, this can be obtained with established tumours.

EXAMPLE 4

A patient suffering from breast cancer was injected with 50μg of the mannan fusion protein produced according to Example 1. No side-effects were seen following immunization. The therapeutic treatment of the patient's cancer is currently under evaluation and it has already been observed that several lesions in the bone have disappeared.

EXAMPLE 5

The VNTR's of mucins MUC1 through MUC7 can be coupled to mannan and other carbohydrates according to standard procedures such as described in Example 1. Table 3 sets out a description of the various mucin core proteins.

TABLE 3: Description of human mucin core proteins, cDNA's and genes

	MUC1	MUC2	MUC3	MUC4	MUC5C	MUC6	MUC7
Tissue	Breast, ovary, pancreas [a]GI, [b]GU and [c]resp. tract	GI and resp.	GI tract	Resp. tract	Trachea Bronchus ± Stomach	Stomach gall bladder	Salivary gland
Polymorphism	Yes	Yes	Yes	?	?	?	?
Chromosome	1q21	11p5.5	7q	3	11p15	11015.4/5	4
Molecular weight of protein kDa	120 - 240	160	80-320	?	?	?	39
Base pairs	60	60	51	48	24	507	69
VNTR* ([d]aa/repeat)	20	23	17	16	8	169	23
Number of repeats	40-80	14	20	39	~20	>6	6

[a]GI - Gastrointestinal; [b]GU - Gastrourinary; [c]Resp. - Respiratory; [d]aa - amino acids

* MUC1 VNTR - SAPDTRPAPGSTAPPAHVT

MUC2 VNTR - PTTTPISTTTMVTPTPTPTGTQT

MUC3 VNTR - HSTPSFTSSITTTETTS

MUC4 VNTR - TSSASTGHATPLPVTD

MUC5B VNTR - 87 base pair degenerate tandem repeats without peptide repeats.

MUC5C VNTR - PTTSTTSA (494 base pair insert - eight amino acid tandem repeat)

MUC6 VNTR - 169aa repeat unit

MUC7 VNTR - TTAAPPTPPATTPAPPSSSAPPE

EXAMPLE 6

MHC restriction of CTL's after MFP immunization

To determine whether the CTL's produced in mice were MHC or indeed, Class I MHC restricted, mice were immunized with MFP (5μg weekly x 3) and their spleen cells removed and used as CTL's against various ^{51}Cr labelled target cells. The results demonstrate that:

a) Immunizing H-2^d mice (DBA/2, NZB, BALB/c or B10.D2) gave CTL's against P815-Tm211 (MUC1$^+$) P815 cells but not against P815-(MUC1) cells.

b) When mice of other H-2 haplotypes were immunized, no CTL's were found on testing the H-2^d P815 MUC1$^+$ cells (in particular H-2^b; C57BL/6; 129 and BALB.B; H-2^k: CBA; H-2^s: SJL and H-2^w: NZW. Of interest in these studies is the finding that:

BALB/c (H-2^d) was +) these are a congenic pair differing only in H-2
BALB.B (H-2^b) was -)

B10.D2 (H-2^d) was +) these are a congenic pair differing only in H-2
C57BL/6 (H-2^b) was -)

This maps the reactivity to the H-2 MHC complex.

c) In other studies it was shown that mice of the H-2^b haplotype had activity for H-2^b (E3 MUC1$^+$ tumour cells), but not for other H-2 haplotypes.

Thus, CTL responses in mice to MFP are H-2 (MHC) restricted.

EXAMPLE 7

T-cell proliferation to MFP in mice

Mice were immunized with various MFP (5μg/week x 3) and tested on a range of peptides at different doses in a proliferation assay. In this assay, different peptides are added in different amounts to splenic cells in tissue culture and after forty-eight hours ^{3}H-thymidine is added for twenty-four hours. The cells are harvested and the incorporated radioactivity measured. The studies show that:

i) MFP stimulates the proliferation of T-cells from immunized mice in the presence of peptides.

ii) There is a dose response so that peptides

C-p13-32, C-p1-24: 5mcM is the optimal dose

p13-32, p1-24: 10mcM is the optimal dose

Ap1-15 < 1.0mcM is the optimal dose

p5-20 < 1.0mcM is the optimal dose

The sequence numbering is such that:

```
1       5         10        15        20 21 next repeat
P D T R P A P G S T A P P A H G V T S A  P ----------
```

iii) Of the peptides used:

p5-20 is +

p14-24) are -

p16-24)

The epitope is not likely to be the antibody epitope APDTR, but is in p14-24 ~ possibly GSTAP.

EXAMPLE 8

MANNAN CONJUGATION WITH ANTIGEN
PREFERENTIALLY INDUCES CTL RESPONSES

Immunotherapy of cancer requires the definition of a suitable target and for solid tumors either the induction of a $CD8^+$ cytotoxic lymphocyte response (CTL) or possibly a DTH (delayed type hypersensitivity) reaction. In breast cancer mucin, MUC1 peptides of the variable number of tandem repeat sequence (VNTR) are a suitable target. The major problem is to induce a cellular immune response. MUC1 peptides conjugated to a carrier (KLH) induce a humoral response and give poor protection in mice with little cellular immunity (that is, a TH_2-type T-cell response). However when MUC1 peptides are conjugated to mannan under oxidizing conditions, a TH_1 response occurs with significant tumor protection, $CD8^+$ CTLs, a high CTLp frequency and little antibody. By contrast, mannan-peptide produced under reducing conditions gives a TH_2-type response. Thus the mode of conjugation (oxidation or reduction) can be used to select the desired type of immune response (TH_1 or TH_2).

A successful vaccine for cancer immunotherapy would require a suitable target antigen and the production of a cytotoxic T-cell response (29). Cancer mucins, particularly MUC1, provide a suitable target in cancer as there is a 10-fold increase in mucin expression, a ubiquitous distribution to all of the cell surface, and altered glycosylation which reveals normally hidden peptide sequences (particularly an antibody epitope: the amino acids APDTR from the variable number of tandem repeat region (VNTR)). These changes generate a new target for immunotherapy which is apparently absent in normal mucin (30). The APDTR sequence is immunogenic in mice leading to antibody formation whether the antigen is administered as $MUC1^+$ cancer cells, purified mucin (HMFG) or peptides (31). Such studies of immunogenicity in mice would be of little relevance to humans, were it not for the findings that tumor specific CTLp exist in the lymph nodes of patients with cancers of either breast, ovary or pancreas (4). These CTLp can be stimulated by antigen and IL-2 *in vitro* to become functional $CD8^+$ CTLs,

the target antigen being either the APDTR sequence of MUC1 or an adjacent sequence in the VNTR (5). The CTLs are unusual in that they have not been shown to be MHC Class I restricted, but can stimulate T-cells, apparently because the multiple repeated epitopes of the VNTR in the MUC1 molecule can crosslink the T-cells receptor (5). Thus, theoretically, patients could be immunized with MUC1 peptide sequences to convert their CTLp into functional CTLs which should have a therapeutic anti-cancer effect. In the present study we show in a murine MUC1$^+$ tumor model, that immunizations with synthetic MUC1 peptide conjugated to a carrier, keyhole limpet hemocyanin (KLH), while being highly immunogenic in terms of antibody production, have little tumor-protective effect. By contrast, a 20mer MUC1 VNTR peptide sequence (made as a GST fusion protein (FP)) when coupled to oxidized mannan (MFP-oxidized) gives protection against MUC1$^+$ mouse tumors and also leads to the rapid reversal of the growth of established MUC1$^+$ tumors; 'reduced' mannan coupled to FP (MFP-reduced) is the same as the peptide-KLH or peptide - DT. These results translate into stimulation of either TH_1 or TH_2 T-cells - depending on the chemical state of the immunogen.

MUC1$^+$ (either MUC1-3T3 or MUC1-P815) mouse tumor cells were produced by transfection of the human MUC1 gene into 3T3 or P815 cell lines (34,35). MUC1-3T3 cells grow as tumors but are rejected by BALB/c mice in ~20 days (due to the immune response to human MUC1); in contrast MUC1-P815 cells grow and are rejected more slowly in ~40 days in DBA/2 mice. Thus there is a "window" between 0 to eleven days in which to observe accelerated rejection or absence of tumor growth in immunized BALB/c animals; this period is longer in DBA/2 mice. In syngeneic mice, MUC1$^+$ tumor rejection is accompanied by the generation of CD8$^+$ CTLs reactive with MUC1$^+$ target cells (36) (See footnote A) (Figure 8A), a high CTLp frequency (36) with a mean of 1/14,600 compared with 1/782,500 in non-immune BALB/c mice (Table 4), little antibody formation (Figure 8B) and DTH mediated by CD4$^+$ cells (Figure 8C) (36). Furthermore such mice are resistant to re-challenge with large doses ($>5 \times 10^7$) of

MUC1$^+$ cells and this is entirely due to conventional H-2 restricted CD8$^+$ cells (36); thus, the ideal type of anti-tumor immunity is to induce CD8$^+$ CTLs and not antibody, a DTH response mediated predominantly by CD4$^+$ cells is present but appears not to be relevant. Several different strategies were used to induce tumor immunity: mice were immunized with either MUC1 synthetic peptides (a 20 amino acid sequence of the VNTR which forms a dimer) coupled to KLH as a carrier (see footnote B), with a MUC1 containing fusion protein produced in the pGEX system consisting of five VNTRs (that is, 100 amino acids) or with natural mucin (HMFG). Three injections calculated to contain 50μg of the mucin component of the immunogen was injected, intraperitoneally at weekly intervals. Each of these procedures induced a similar immune response, and the data for fusion protein suffice to illustrate this. These procedures led to some degree of protection when the mice were challenged with 10^6 MUC1-3T3 cells when tumors had a delayed appearance and were smaller (Figure 9A), but not when the challenge dose was increased to 5 x 10^6 cells (Figure 9B). In each case no CTLs could be detected (Figure 8A), but there was high antibody production (Figure 8B), that is, a typical TH2-type response. The tumor protection observed by challenging mice with 10^6 cells may have been mediated by the few CTL (note the low CTLp frequency of 1/87,400 (50μg) or 1/150,000 (5μg) (Table 14)), which were too few to detect in the standard CTL assay which directly analyzed freshly isolated splenocytes.

To alter the immunogenicity of the MUC1 peptides aimed to induce a TH_1-type response similar to that induced directly by tumor cells (that is, a predominant CD8$^+$ CTL response), various protocols were used, the most successful being the conjugation of the fusion protein to mannan, a carbohydrate carrier previously used to increase immune responses. In the past mannan was used to induce cellular immunity to murine candidiasis, to enhance the action of lipopolysaccharides on T-cells proliferation against protein antigens, and to induce anti-peptide antibody responses (37, 38, 39). The optimal immunogenic preparation (MFP) consisted of the MUC1 fusion protein (FP)

with GST attached, coupled by Schiff bases to aldehyde groups on oxidized (periodate treated) mannan (see footnote C). It was found to be critical in the procedure that the Schiff bases and residual aldehyde groups were not reduced with borohydride. Mice were injected with MFP (5μg of the FP component three times intraperitoneally at weekly intervals) and analyzed for their immune status prior to tumor challenge: there were $CD8^+$ CTLs present (Figure 8A), a high CTLp frequency with a mean of 1/6,900 compared with 1/782,500 in non-immune BALB/c mice (Table 4), no detectable antibody titre (Figure 8B), but significant DTH responses (Figure 8C). We believe this is the first description of tumor cell killing generated with synthetic MUC1 antigens. When challenged with tumor cells, total protection occurred, even up to doses of 50 x 10^6 cells (Fig. 2A) (the largest dose which could be given) that is, at least fifty times the protection afforded by immunization with FP or free peptide. A number of controls for cell target and immunization specificity were used and no protection was observed with: a) mannan and FP were mixed (non-conjugated), b) mannan was conjugated to another irrelevant fusion protein or c) mannan was injected alone (Fig. 3) Clearly, the MUC1 FP must be conjugated to mannan to immunise against $MUC1^+$ tumors and the crucial part of the linkage procedure was to leave the MFP in the oxidized state to preserve the aldehyde groups and the Schiff bases. When non-oxidized MFP was used (that is, reduced with sodium borohydride) there neither was tumor protection nor were CTLs generated (Figure 8A and 8B, Table 4). Indeed, reduced MFP stimulated immunity similar to FP or peptide not conjugated to mannan.

Immunizing mice prior to tumor cell challenge is not a realistic model for the treatment of patients who already have cancer (although, it could be feasible for future studies with MFP as a "vaccine" to immunise patients in remission who do not have tumors). Thus, mice with established MUC1-P815 tumors were treated once or three times with MFP (5μg of FP antigen) given on days fifteen, seventeen and nineteen after tumor injection. After a single injection, rapid reversal of progressive tumor growth occurred within four days, and after several injections there was a rapid and early disappearance

of the tumors, indicating that MFP is highly immunogenic, even in the presence of a substantial tumor load.

Immunization with MUC1 peptides conjugated to mannan gives rise to $CD8^+$ tumor specific cytotoxic T-cells responses, similar to the response naturally produced to tumors and which is responsible for tumor rejection. Although not yet proven, we consider the mannan is likely to target antigens to mannose receptors on macrophages. Indeed, it is the splenic sinusoidal cells in marginal sinuses which have the highest density of receptors for mannose (40). Furthermore, the fusion protein when similarly linked under oxidizing conditions to dextran (a polysaccharide which lacks mannose residues) could not induce antitumor immunity (not shown). However, targeting to macrophages alone is insufficient: the MFP needs to be in oxidized form, probably due to the requirement for aldehyde groups which are considered to be important in the interaction of T-cell glycoproteins (for example, the T-cells receptor) and glycoproteins on the antigen presenting cells (41). Thus, oxidized MFP is likely to do two things: a) the mannan specifically targets the antigen to macrophages, and b) in oxidized form the formation of Schiff bases augments interactions required for T-cells activation. We note that anti-peptide antibodies have been made by conjugating a peptide using cyanogen bromide (37, 38, 39); this would yield a product analogous to the reduced MFP described above - that is, findings in accord with our results with a TH_2-type response.

In mice, oxidized MFP is clearly a superior immunization method for MUC1 synthetic peptides when CTLs are required. It is of interest that if MFP is reduced with sodium borohydride, then antibody responses preferentially occur, similar to using the peptide - carrier immunization described herein and in (36). Thus, at will, we can produce either CTL responses by oxidation or antibody responses by reduction of the same product. Although individual cytokines were not measured, it appears that oxidized mannan-antigen immunization preferentially stimulates a $CD8^+$ TH_1-type response,

whereas the reduced form stimulates the production of antibodies, and alters to a TH_2-type response. Whether this can be a standard approach applied to other peptides is currently under examination and whether cancer patients can be induced to make an effective immune response to MUC1 conjugated to mannan and whether this is immunogenic enough to generate sufficient functional CTLs to cause cancer regression in cancer patients will require appropriate clinical trials.

Footnote A

Cytotoxic T lymphocyte precursor cell (CTLp) frequency determinations : For each cell suspension in which CTLp frequencies were determined, a minimum of thirty two replicates for each of at least six responder cell doses were cultured in U-bottomed microtiter trays, with 5 x 10^5 BALB/c spleen stimulator cells in modified Eagle's medium supplemented with 10% fetal calf serum, with 5μM synthetic MUC1 peptide and 10μ/ml rIL-2. Seven days later, each microculture was assayed for cytotoxicity by replacing 100μl of culture medium with 100μl target cell suspension containing 10^4 ^{51}Cr-labelled MUC1-P815 tumor target cells. Wells were regarded as containing cytotoxic activity if they yielded specific ^{51}Cr release three standard deviation above the mean isotope release from 10^4 cells cultured alone or with stimulators only, peptide only or rIL-2 only. A linear relationship existed between the dose of responder cells on a linear scale and the frequency of negative wells on a logarithmic scale. CTLp frequencies were determined as the inverse of the inverse of responder cell dose required to generate 37% negative wells

Footnote B

Peptides were synthesized on an Applied Biosystems synthesizer. The sequence of the synthetic MUC1 VNTR was C-PDTRPAPGSTAPPAHGVTSA; the C added to N-terminal aided in conjugation and enabled homodimers to form. This peptide was conjugated to Keyhole limpet hemocyanin using glutaraldehyde (36). The fusion proteinconsisted of five VNTRs (that is, 100 amino acids) produced in the pGEX expression system and cleaved with GST attached (9).

Footnote C

Mannan (Sigma), 14mg/ml in 0.1M phosphate buffer pH6.0, was oxidized with sodium periodate (0.01M) for 30-60 minutes at 4°C. Ethandiol (10μl) was added and incubated for a further thirty minutes at 4°C and passed through a Sephadex-G25 column equilibrated in bicarbonate buffer pH6.0 to pH9.0. The oxidized mannan that eluted in the void volume (2mls) was mixed with 900μg of MUC1 fusion protein and incubated overnight at room temperature and used without further purification. To form reduced MFP the mixture was treated with 1mg/ml sodium borohydride for three hours. The purity of MFP was analyzed by SDS-PAGE and gel permeation chromatography

TABLE 4: CTLp frequency in mice immunized with different immunogens:

Immunogens	CTLp Frequency*			
	Mouse #1	Mouse #2	Mouse #3	Mean
Non-immunized				
BALB/c mice	1/850,000	1,725,000		1/782,500
Mice immunized with:				
Tumour cells	1/12,500	1/17,500		1/14,600
MFP-oxidized	1/8,800 **	1/6,200	1/6,300	1/6,900
MFP-reduced	1/84,000	1/92,000		1/87,800
FP (50μg)	1/85,000	1/90,000		1/87,400
FP (5μg)	1,150,000			
Cp13-32-KLH	1/150,000	1,150,000		1/150,000
HMFG	1/340,000	1/440,000		1/383,700

* Analysis of individual BALB/c female mice.

** CTLp frequency of two pooled BALB/c female mice.

EXAMPLE 10

CTL IN MICE IMMUNIZED AGAINST HUMAN MUC1 ARE MHC RESTRICTED

$CD8^+$ cytotoxic T-cells have been induced to endogenous peptides associated with MHC Class I molecules, the interacting complex also requiring reaction of T-cells receptor α, β reacting with the antigenic peptide in the groove of the MHC. Such CTL's have been produced to endogenous peptides or exogenous infectious agents particularly viral peptides, all of which cycle through well described intracellular pathways and while it has been difficult to induce CTL's to exogenous peptides too, it is certainly well described, in viral infections such as influenza, EBV and the like. It is the aim of many cancer immunotherapy studies to induce such CTL's reacting against tumour peptides. In melanoma in humans such CTL's have also been MHC restricted associating particularly with HLA-A1 and HLA-A2. However, recently a new type of CTL was described by O. Finn and colleagues, the description giving great impetus to immunizing humans against solid tumours of breast, pancreas and ovary. What was described was the presence, in the draining lymph nodes of women with breast cancer, precursors to CTL's which could *in vitro* be stimulated in the presence of IL-2 and different breast cancer cell lines to develop CTL's to breast cancer cell lines. The cells were $CD3^+$ $CD8^+$, but reacted in a non-MHC restricted manner to lyse target cells. The findings were not unique to breast cancer and have subsequently been found in draining lymph nodes of patients with carcinomas of either the pancreas, ovary and more recently in the circulation of patients with multiple myeloma. The CTL's react with MUC1 molecules, indeed with the repeating peptide unit (found as a variable number of tandem repeats, but in the protein core) as antibodies to the highly immunogenic APDTR region of the peptide core block the reaction of the CTL's on $MUC1^+$ target cells. Furthermore, the specificity of the CTL's was confirmed by reactivity with $MUC1^+$ target cells produced by transfection and non-reactivity on non-

MUC1 cells. Although the presence of non-MHC restricted CTL's defied convention, their mode of action is likely to be by cross-linking of the T-cell receptor by the repeating units on MUC1 rather than requiring a CD8/Class I interaction, and compelling evidence for such cross-linking has recently been provided. With the observations that MUC1 is immunogenic in mice for the induction of antibodies and appears to be immunogenic in humans to induce CTL's, we have embarked upon a program with the preclinical immunization of mice with soluble recombinant MUC1 molecules and a clinical trial has commenced with such materials. $MUC1^+$ murine tumours (produced by transfection) are readily rejected by mice with the induction of memory T-cells (resistant to a challenge), little antibody and CTL response; Immunization with MUC1 peptides, however, produces a predominate antibody response without CTL's. By conjugation of the fusion protein with mannan a significant CTL response can be induced in mice and we now report the CTL response is of the conventional type in that it is mediated by $CD3^+$ $CD8^+$ cells which are MHC restricted - indeed, MUC1 molecules appear to be able to associate with at least seven or eight different types of Class I molecules, but the association is necessary for CTL's to function.

Immunizing mice with human MUC1 (MFP) leads to MHC restricted CTL's. These were found in mice with haplotypes b, d, k, z and s and were clearly MHC restricted as shown in congenic strains. Further testing in recombinant strains demonstrated MUC1 peptides are able to associate with D or K Class I molecules of the b, d or k haplotype. Clearly, anti-MUC1 CTL's induced by immunization are different from those which arise spontaneously in patients with tumours and should be borne in mind in clinical studies with a view to immunizing patients against their tumours.

A summary of the H-2 restriction studies is shown in Table 5 and results are shown in Figures 10 to 20.

TABLE 5: Summary of the H-2 restriction studies

Immunised mouse strain	H-2	Target cell (MUC1 + cell line or peptide pulsed blast)	Target cell lysis
a) INBRED LINES C57BL/6, 129J	b	bC57BL/6 (spleen or E3)	+
	b	d BALB/c, B10.D2,NZB	-
BALB/c, DBA/2, B10.D2	d	d BALB/C, B10.D2, NZB	+
NZB		B C57BL/6	-
CBA	k	k CBA b a above d as above	+ - -
SJL	s	s SJL z NZW d as above	+ - -
NZW	z	z NZW s SJL d B10D2	- - -
b) CONGENIC C57BL/6	b	b C57BL/6 d B10.D2	+ -
BALB/c	d	d BALB/c b BALB/B	+ -
B10.D2	d	d BALB/c	+
	d	b C57BL/6	-
c) RE-COMBINANTS b10.a 92r)	K^kD^b	K^kD^bB10.D2 K^kD^b C57BL/6 K^kD^b B10.D2 K^kD^d B10.D2	+ + + -
B10.A (5R)	K^kD^d	K^kD^b 5R K CBA K^kD^b C57BL/6 d BALB/c	+ - + +

Immunised mouse strain	H-2	Target cell (MUC1 + cell line or peptide pulsed blast)	Target cell lysis
(d) MUTANTS bm-1 (class I mutant)	KbmDb	K^kD^b(bm1) K^kD^b K^kD^b K^kD^d (bm-12) K^kD^b D^d	+ + - + + -
bm-12(class II mutant)	KbmDb	K^kD^b(bm1) K^kD^b K^kD^d K^kD^b(bm-12) K^kD^b D^d	+ + + + + + -
β2M-/-	K^kD^b	K^bD^b(β2m-/-) K^kD^b K^kD^d K^kD^b K^bD^b(βm-1) D^d z	+ + + + + + -

EXAMPLE 11

THE IMMUNOGLOBULIN ISOTOPE OF ANTIBODIES

The immunoglobulin isotope of Antibodies produced by the various immunogens was determined by an ELISA.

Mice immunized with:

FP	had IgG1, IgG2a, IgG2b, IgM antibodies Predominantly IgG1
Cp13-32	had IgG1 and IgM antibodies Predominantly IgG1
HMFG	had IgM antibodies

It should be noted that in the tumour protection studies the protection was:

FP > Cp13-32 > HMFG and this correlated with their CTLp frequencies. It seems that IgM gives the least protection and then IgG1. Where all isotopes (including IgG2a) were produced the protection and CTLp frequency was better (as seen be immunizing with FP).

Mice immunized with:

Tumour cells	no antibodies were produced
MFP-oxidized	had very little antibody produced and this was of the IgG2a isotope

The above two immunogens gave the best protection, high CTLp frequencies and cellular immunity (presumably of the TH_1 type). Immunizing with FP, Cp13-32 and HMFG gives humoral immunity (antibodies and no cytotoxic T-cells) - presumably of the TH_1 type.

Mice immunized with:

MFP-reduced IgG1 antibodies were produced.

This appears to be of TH2 type response (no CTL were found) (Example 9).

Thus depending on the mode of MFP (either reduced of oxidized) we are able to produce cellular or humoral immunity.

Figure showing Antibody production in mice immunized with the different immunogens is Figure 20.

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The claims defining the invention are as follows:

1. A compound comprising a conjugate between an antigen, and a carbohydrate polymer.

2. A compound according to claim 1, wherein said antigen is selected from pollen, hepatitis C virus (HIV) core, E1, E2 and NS2 proteins, Plasmodium falciparum circumsporozoite protein, HIV-gp120/160 envelope glycoprotein, streptococcus surface protein Ag, influenza nucleoprotein, haemagglutinin-neuraminidase surface infection, TcpA pilin subunit, VP1 protein, LMCV nucleoprotein, Leishmania major surface glycoprotein (gp63), Bordetella pertussis surface protein, rabies virus G protein, Streptococcus M protein, Syncyticial virus (RSV) F or G proteins, Epstein Barr virus (EBV) gp340 or nucleoantigen 3A, haemagglutinin, Borrelia burgdorferi outer surface protein (Osp) A, Mycobacterium tuberculosis 38kDa lipoprotein or Ag85, Neisseria meningitidis class 1 outer protein, Varicella zoster virus IE62 and gpI, Rubella virus capsid protein, Hepatitis B virus pre S1 ag, Herpes simplex virus type I glycoprotein G or gp D or CP27, Murray valley encephalitis virus E glycoprotein, Hepatitis A virus VP1, polio virus capsid protein VP1, VP2 and VP3, chlamydia trachomatis surface protein, Hepatitis B virus envelope Ag pre S2, Human rhinovirus (HRV) capsid, papillomavirus peptides from oncogene E6 and E7, Listeria surface protein, Varicella virus envelope protein, Vaccinia virus envelope protein, Brucella surface protein, a combination of one or more of said antigens, an amino acid subunit of said antigens comprising five or more amino acids in length or combinations of one or more of said subunits.

3. A compound according to claim 1, wherein said antigen is the human mucin polypeptide, one or more repeated subunits thereof, or a fragment of said repeated subunits.

4. A compound according to claim 3 comprising two to eighty copies of the repeated subunits of said human mucin.

5. A compound according to either claim 2 or claim 3, wherein said one or more repeated subunits of said antigen comprise part of a fusion polypeptide.

6. A compound according to any one of claims 1 to 5, wherein said carbohydrate polymer is a polymer of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhaminose, 6-0-methyl-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose or α abequose, conformational and configurational isomers thereof, or a carbohydrate polymer comprised of two or more of said carbohydrate units.

7. An immunogenic vaccine against disease states which comprises a compound comprising a conjugate between an antigen and a carbohydrate polymer, in association with a pharmaceutically acceptable carrier.

8. An immunogenic vaccine as claimed in claim 7, wherein said antigen is an antigen as defined in claim 2.

9. An immunogenic vaccine as claimed in claim 7, which is directed against tumour cells expressing human mucin or a subunit thereof, and wherein said

antigen comprises the human mucin polypeptide, one or more repeated subunits thereof, or a fragment of said repeated subunits.

10. An immunogenic vaccine according to claim 9, wherein said conjugate comprises two to eighty copies of the repeated subunits of said human mucin.

11. An immunogenic vaccine according to either claim 8 or claim 9, wherein said one or more subunits repeated subunits of said antigen comprise part of a fusion polypeptide.

12. An immunogenic vaccine according to any one of claims 7 to 11, wherein said carbohydrate polymer is a polymer of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhaminose, 6-0-methyl-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose or α abequose, conformational and configurational isomers thereof, or a carbohydrate polymer comprised of two or more of said carbohydrate units.

13. A method for inducing a cell-mediated immune response against an antigen, which comprises administering to an animal a compound comprising a conjugate between said antigen, and a carbohydrate polymer, optionally in association with a pharmaceutically acceptable carrier.

14. The method of claim 13, wherein said antigen is an antigen as defined in claim 2.

15. The method of claim 13, wherein said cell-mediated immune response is induced against cells expressing human mucin, or subunits thereof, and wherein said human antigen comprises the human mucin polypeptide.

16. The method according to claim 15, wherein said conjugate comprises from two to eighty copies of the repeated subunit of said human mucin.

17. A method according to either claims 14 or 15, wherein said one or more subunits repeated subunits of antigen comprise part of a fusion polypeptide.

18. A method according to any one of claims 13 to 17, wherein said carbohydrate polymer is a polymer of glucose, galactose, mannose, xylose, arabinose, fucose, glucosamine, galactosamine, rhaminose, 6-0-methyl-D-galactose, 2-0-acetyl-β-D-xylose, N-acetyl-glucosamine, iduronate, guluronate, mannuronate, methyl galacturonate, α-D-galactopyranose 6-sulphate, fructose or α abequose, conformational and configurational isomers thereof, or a carbohydrate polymer comprised of two or more of said carbohydrate units.

19. A compound according to claim 6, wherein said carbohydrate polymer is mannan.

20. An immunogenic vaccine according to claim 12, wherein said carbohydrate polymer is mannan.

21. A method according to claim 18, wherein said carbohydrate polymer is mannan.

22. A compound according to either claim 3 or claim 4, wherein said human mucin is MUC1.

23. An immunogenic vaccine according to either claim 9 or claim 10, wherein said human mucin is MUC1.

24. A method according to either claim 15 or claim 16, wherein said human mucin is MUC1.

25. A compound according to claim 1, wherein said antigen comprises whole cells or sub-cellular fractions thereof.

26. An immunogenic vaccine according to claim 7, wherein said antigen comprises whole cells or sub-cellular fractions thereof.

27. The method of claim 13, wherein said antigen comprises whole cells or sub-cellular fractions thereof.

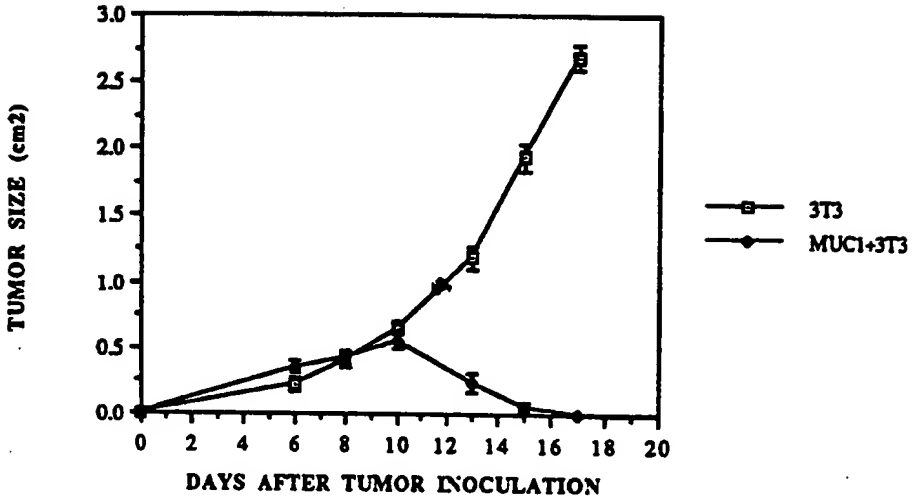
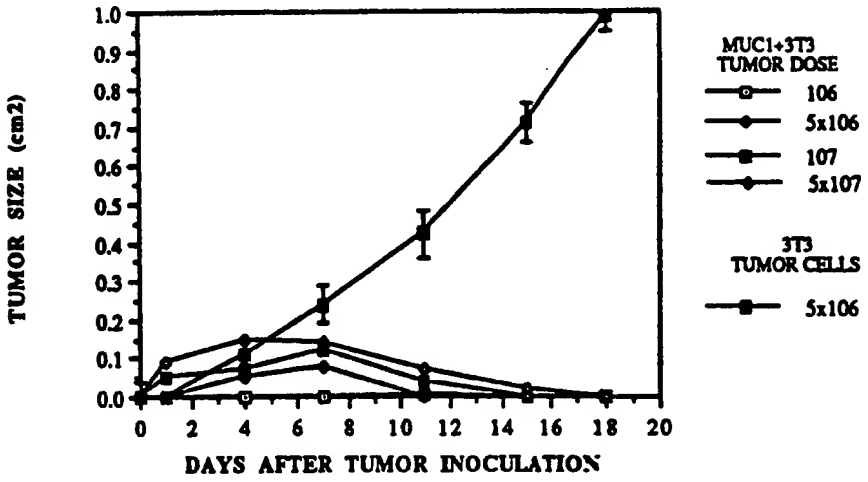

TUMOR SIZE (cm2)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
18
20
DAYS AFTER TUMOR INOCULATION
3T3
MUC1+3T3

Fig.1

A


Tumor challenge in Mannan-FP immunized mice
TUMOR SIZE (cm2)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 2 4 6 8 10 12 14 16 18 20
DAYS AFTER TUMOR INOCULATION
MUC1+3T3 TUMOR DOSE
106
5x106
107
5x107
3T3 TUMOR CELLS
5x106

B

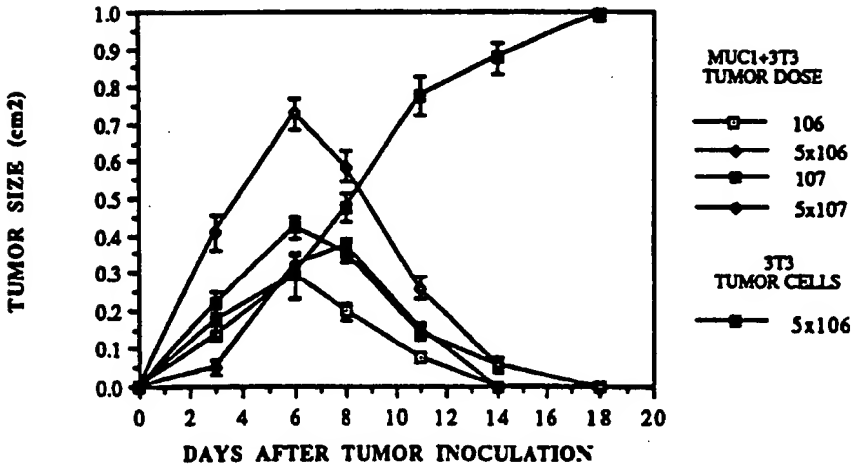
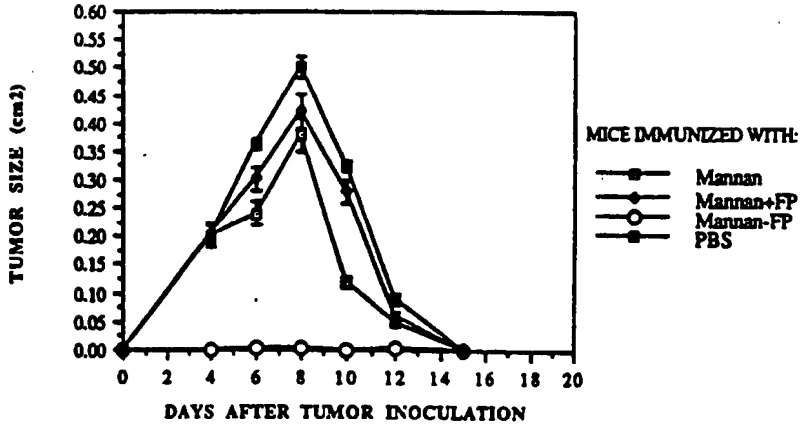

Tumor challenge in control mice
TUMOR SIZE (cm2)
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 2 4 6 8 10 12 14 16 18 20
DAYS AFTER TUMOR INOCULATION
MUC1+3T3 TUMOR DOSE
106
5x106
107
5x107
3T3 TUMOR CELLS
5x106

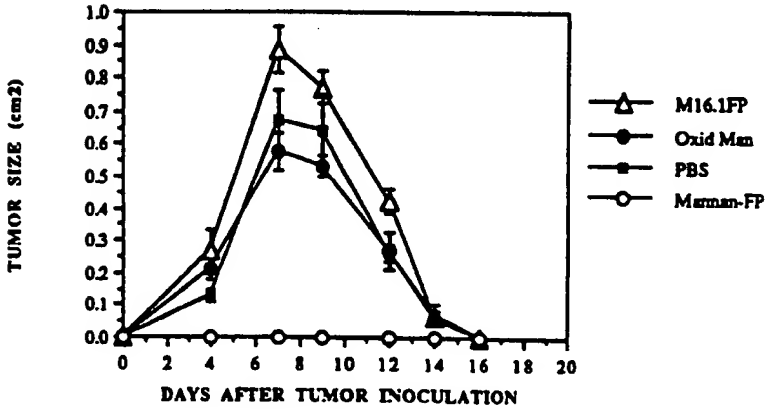
Fig.2

Tumor challenge with 10 6 cells

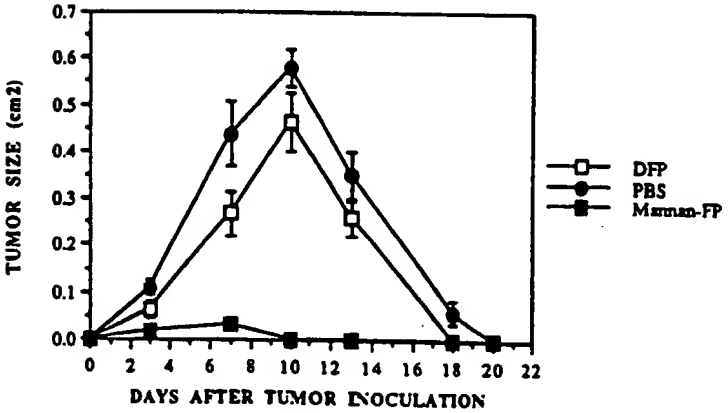
A



B



C



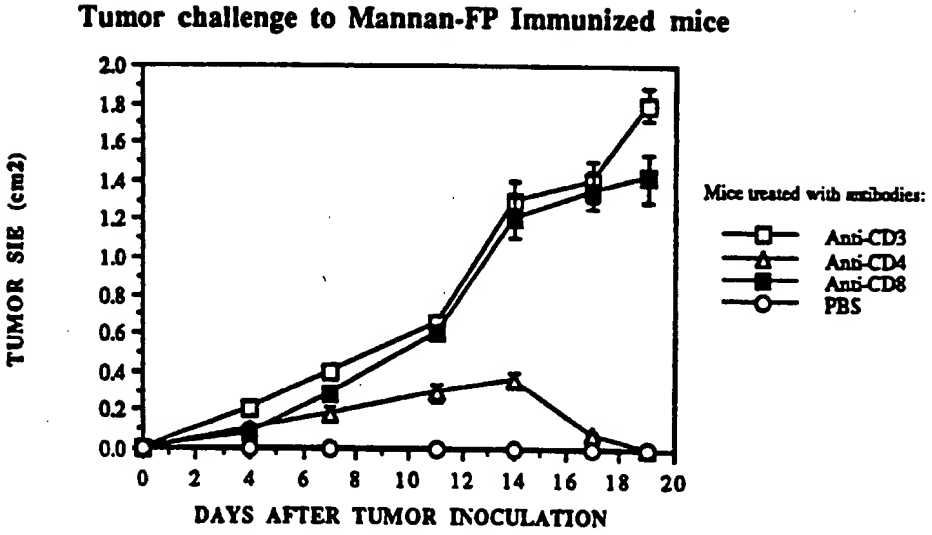

Tumor challenge to Mannan-FP Immunized mice
TUMOR SIE (cm2)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0 2 4 6 8 10 12 14 16 18 20
DAYS AFTER TUMOR INOCULATION
Mice treated with antibodies:
Anti-CD3
Anti-CD4
Anti-CD8
PBS

Fig.4

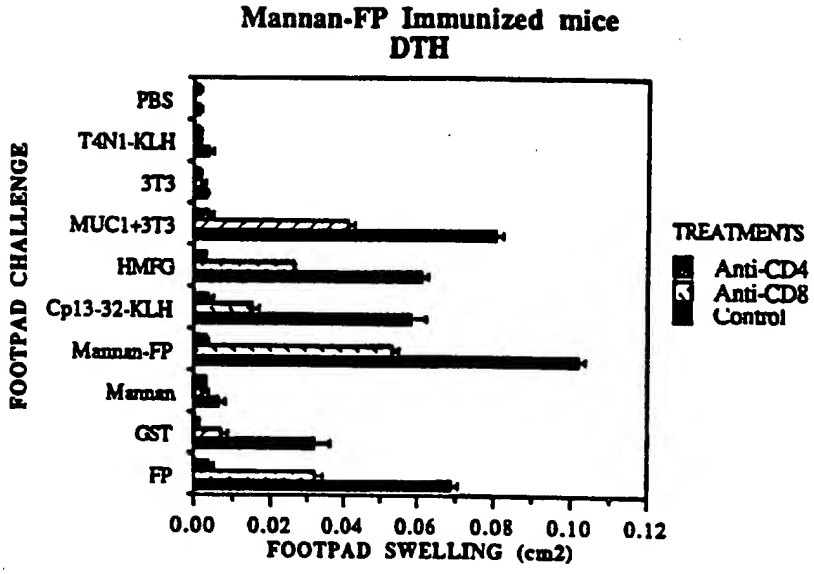

Mannan-FP Immunized mice
DTH
FOOTPAD CHALLENGE
PBS
T4N1-KLH
3T3
MUC1+3T3
HMFG
Cp13-32-KLH
Mannan-FP
Mannan
GST
FP
0.00
0.02
0.04
0.06
0.08
0.10
0.12
FOOTPAD SWELLING (cm2)
TREATMENTS
Anti-CD4
Anti-CD8
Control

Fig.5

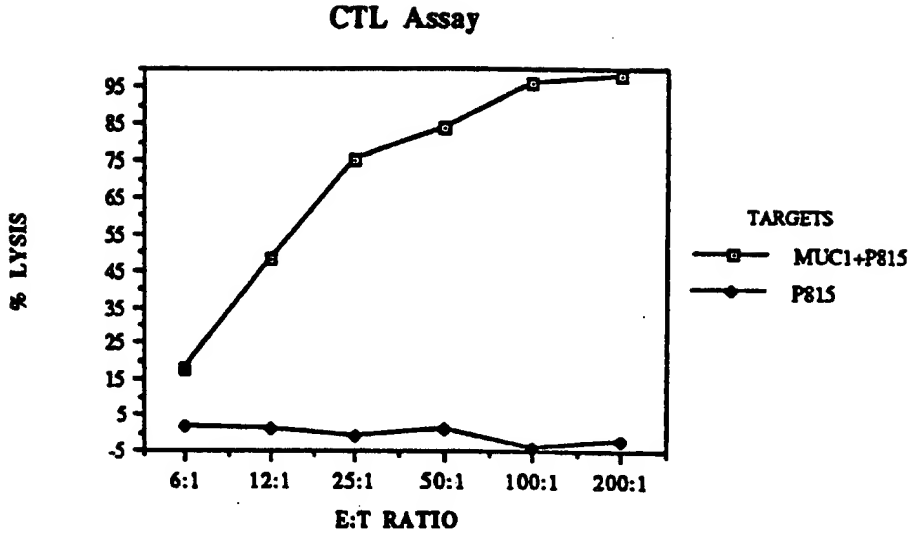
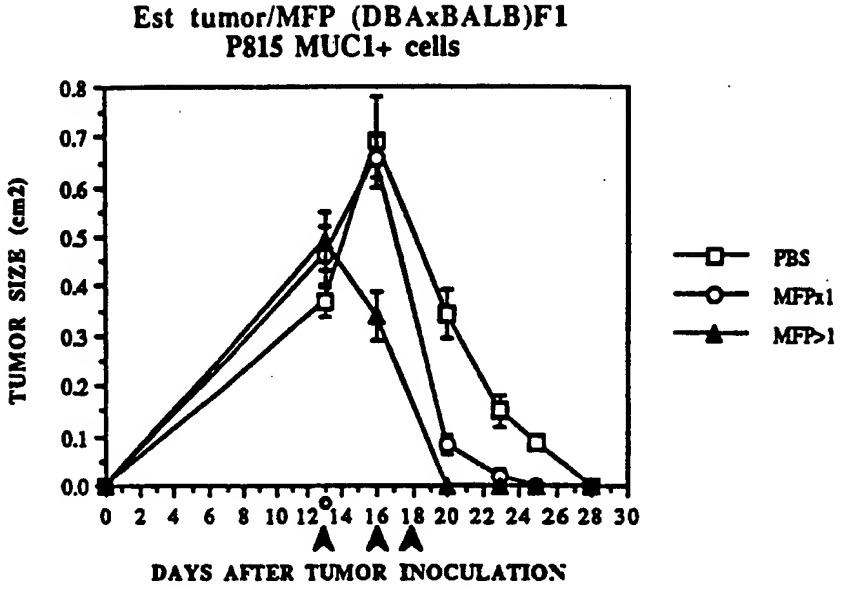

CTL Assay
% LYSIS
95
85
75
65
55
45
35
25
15
5
-5
6:1
12:1
25:1
50:1
100:1
200:1
E:T RATIO
TARGETS
MUC1+P815
P815

Fig.6

A


Est tumor/MFP (DBAxBALB)F1
P815 MUC1+ cells
TUMOR SIZE (cm2)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
DAYS AFTER TUMOR INOCULATION
PBS
MFPx1
MFP>1

B

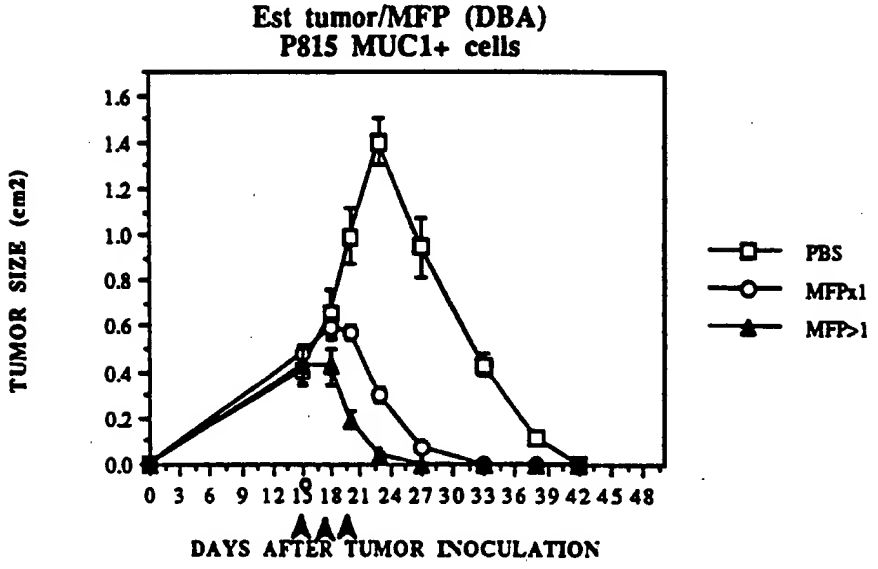

Est tumor/MFP (DBA)
P815 MUC1+ cells
TUMOR SIZE (cm2)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0 3 6 9 12 15 18 21 24 27 30 33 36 39 42 45 48
DAYS AFTER TUMOR INOCULATION
PBS
MFPx1
MFP>1

Fig.7

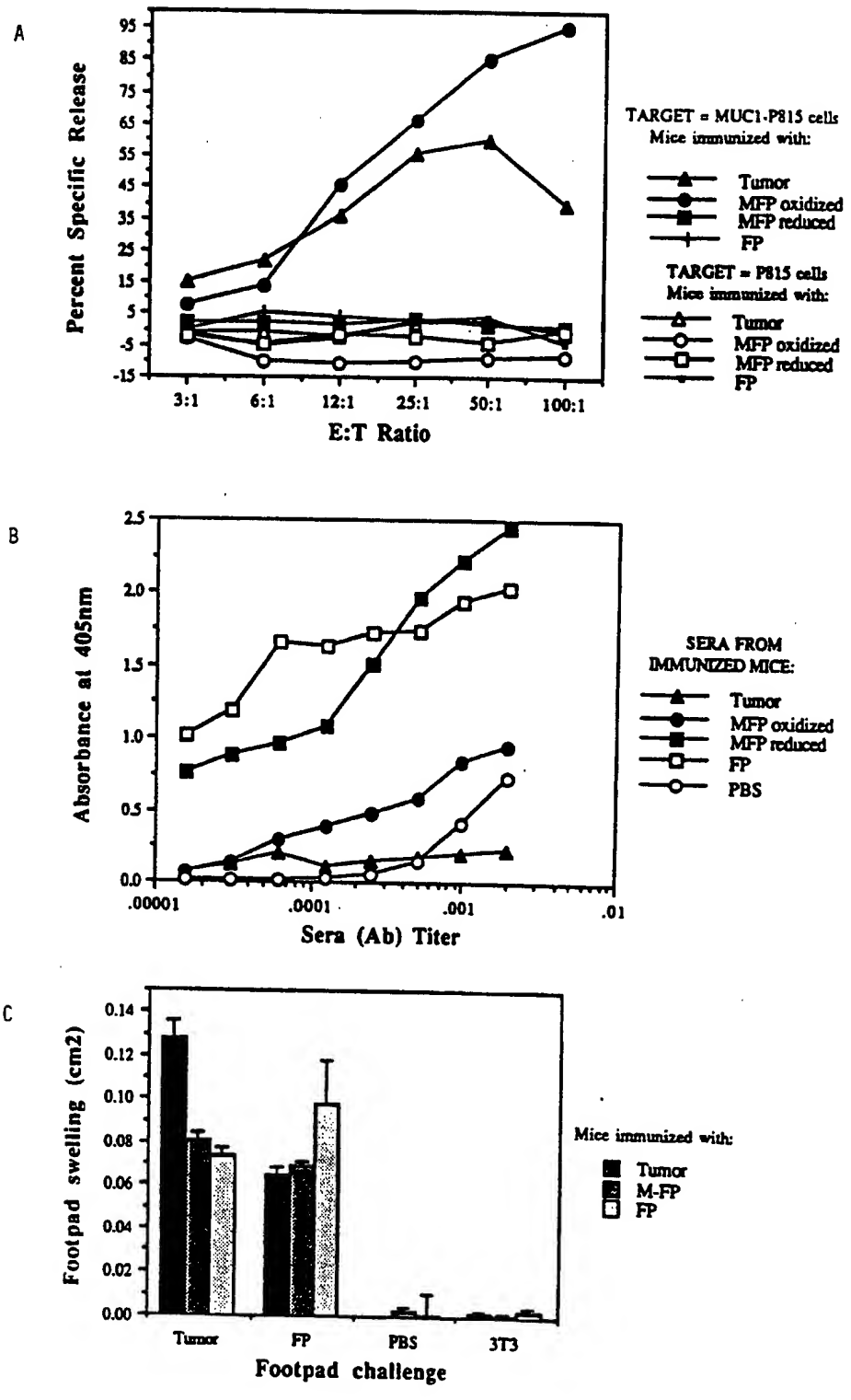

A
Percent Specific Release
95
85
75
65
55
45
35
25
15
5
-5
-15
3:1
6:1
12:1
25:1
50:1
100:1
E:T Ratio
TARGET = MUC1-P815 cells
Mice immunized with:
Tumor
MFP oxidized
MFP reduced
FP
TARGET = P815 cells
Mice immunized with:
Tumor
MFP oxidized
MFP reduced
FP
B
Absorbance at 405nm
2.5
2.0
1.5
1.0
0.5
0.0
.00001
.0001
.001
.01
Sera (Ab) Titer
SERA FROM
IMMUNIZED MICE:
Tumor
MFP oxidized
MFP reduced
FP
PBS
C
Footpad swelling (cm2)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Tumor
FP
PBS
3T3
Footpad challenge
Mice immunized with:
Tumor
M-FP
FP

Fig.8

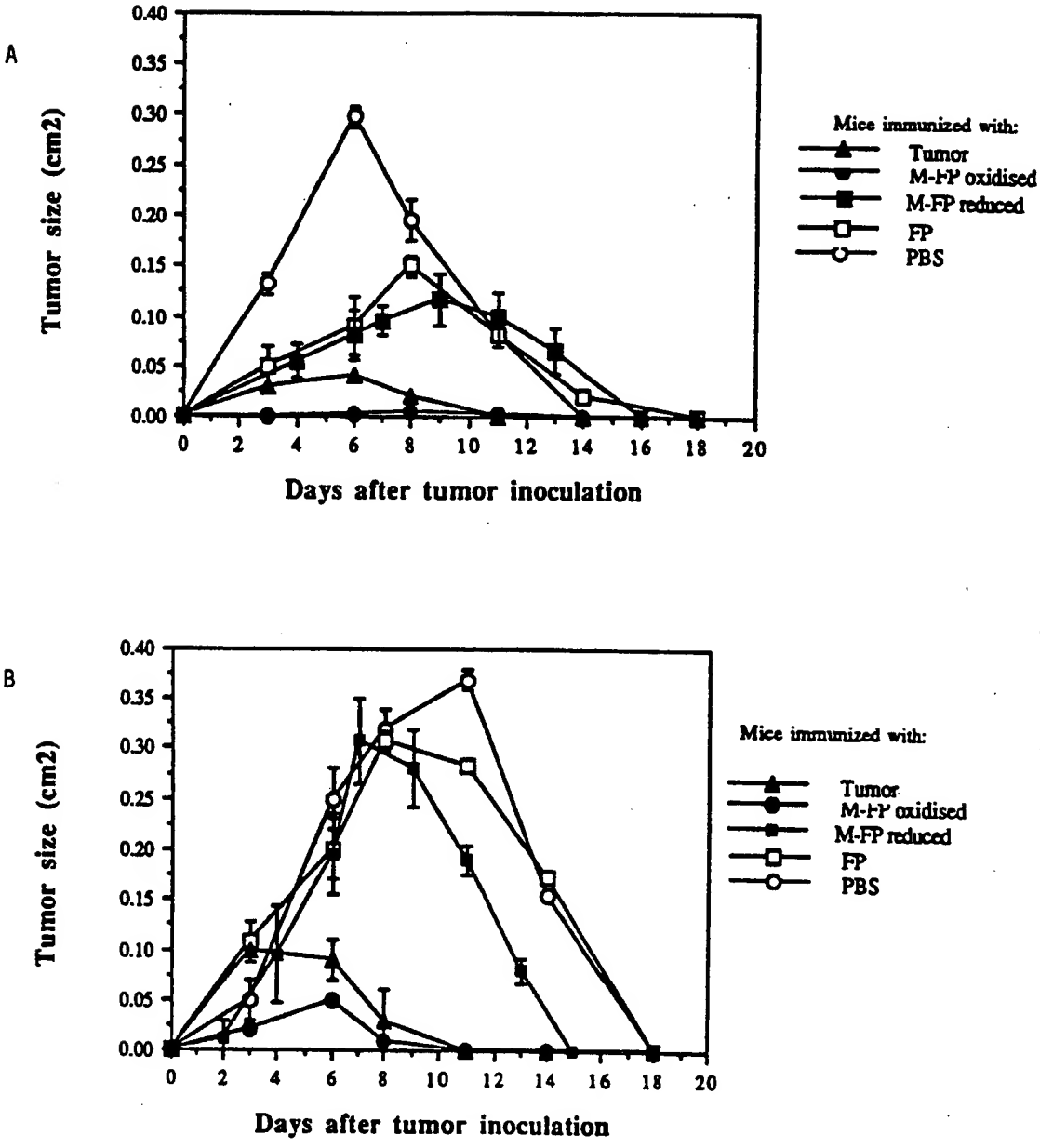
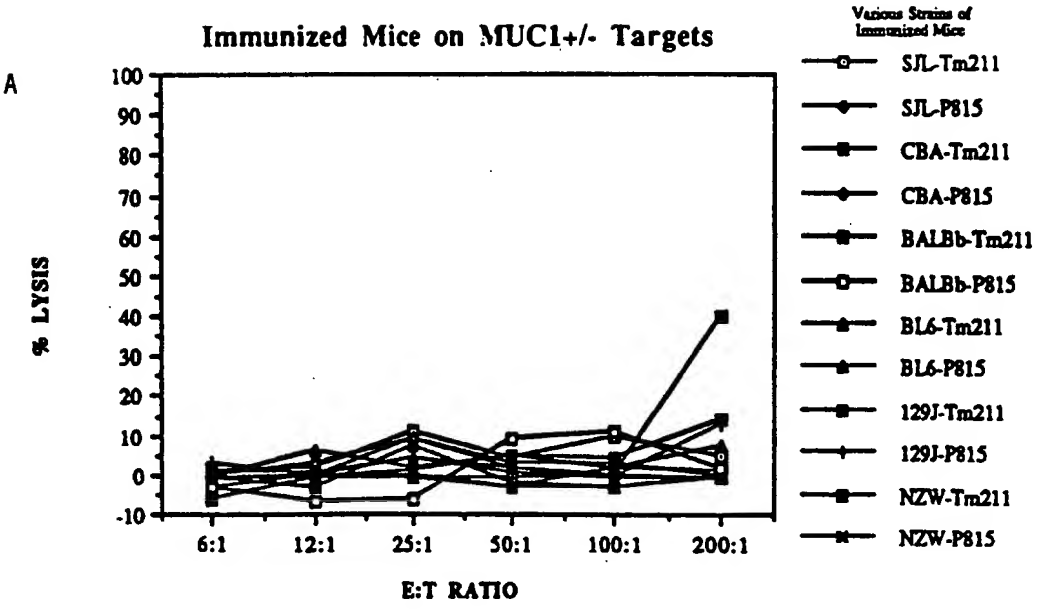

A
Tumor size (cm2)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 2 4 6 8 10 12 14 16 18 20
Days after tumor inoculation
Mice immunized with:
Tumor
M-FP oxidised
M-FP reduced
FP
PBS
B
Tumor size (cm2)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0 2 4 6 8 10 12 14 16 18 20
Days after tumor inoculation
Mice immunized with:
Tumor
M-FP oxidised
M-FP reduced
FP
PBS

Fig.9

A


Immunized Mice on MUC1+/- Targets
% LYSIS
E:T RATIO
6:1
12:1
25:1
50:1
100:1
200:1
Various Strains of Immunized Mice
SJL-Tm211
SJL-P815
CBA-Tm211
CBA-P815
BALBb-Tm211
BALBb-P815
BL6-Tm211
BL6-P815
129J-Tm211
129J-P815
NZW-Tm211
NZW-P815

B

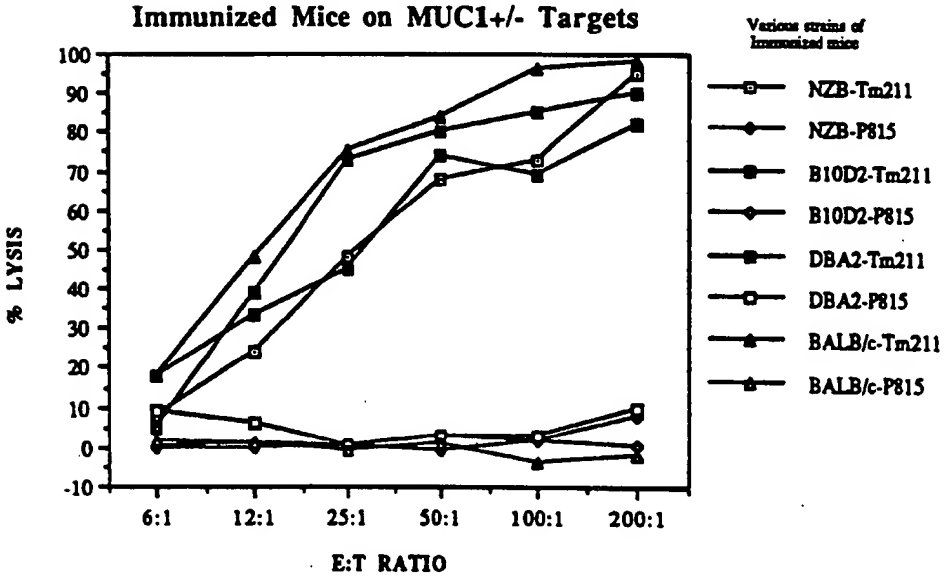
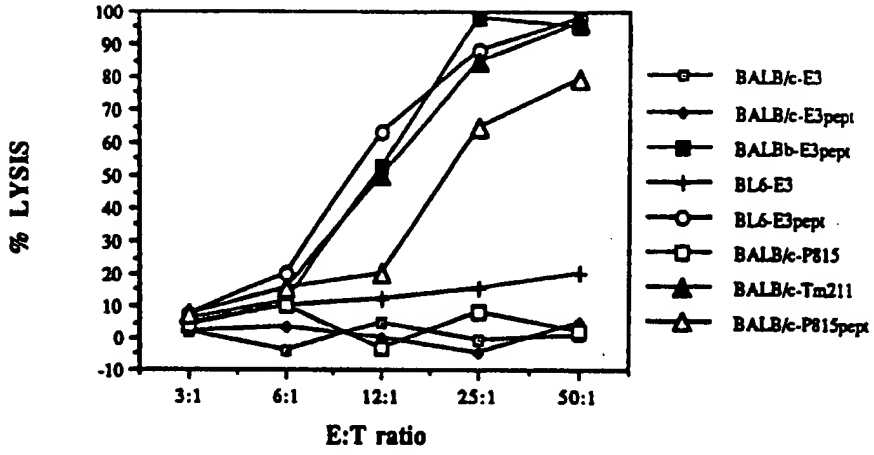

Immunized Mice on MUC1+/- Targets
% LYSIS
E:T RATIO
6:1
12:1
25:1
50:1
100:1
200:1
Various strains of Immunized mice
NZB-Tm211
NZB-P815
B10D2-Tm211
B10D2-P815
DBA2-Tm211
DBA2-P815
BALB/c-Tm211
BALB/c-P815

Fig.10

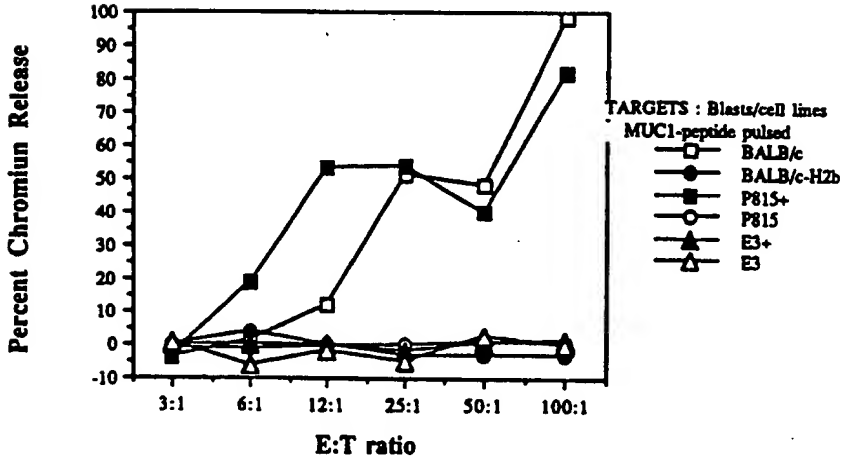
H2 Restriction using E3-peptide

A



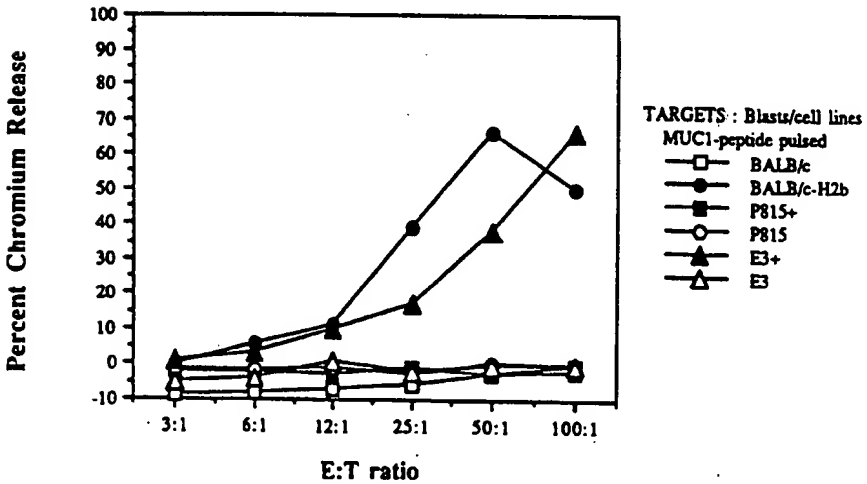
BALB/c Immunized mice

B



BALB/c-H2b Immunized mice

C



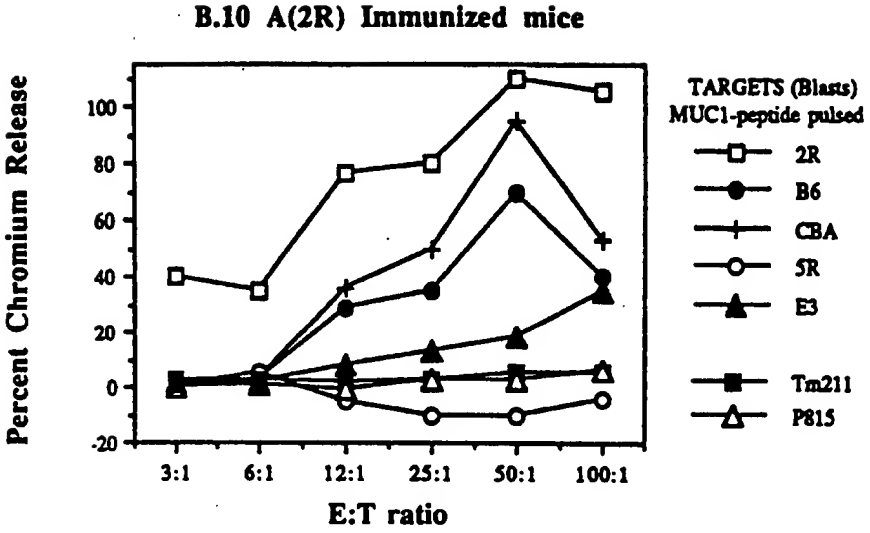

B.10 A(2R) Immunized mice
Percent Chromium Release
100
80
60
40
20
0
-20
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
2R
B6
CBA
5R
E3
Tm211
P815

Fig.12

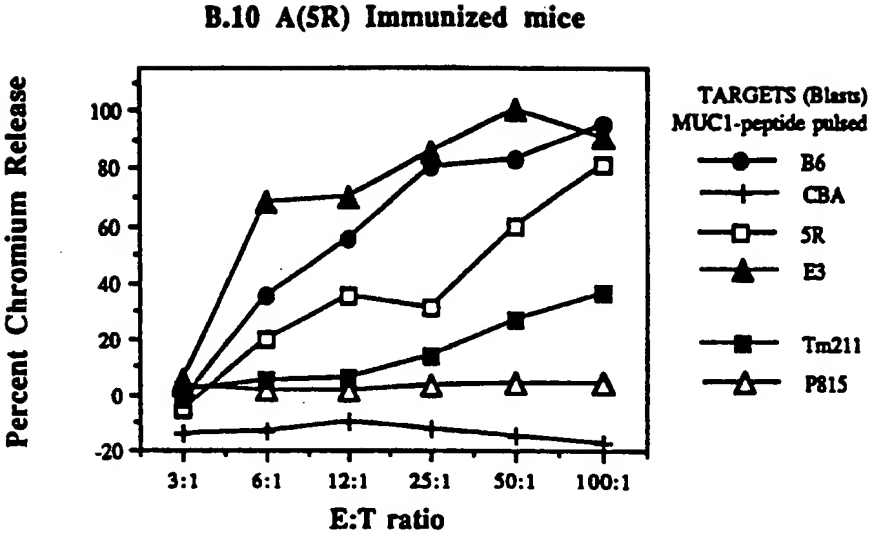

B.10 A(5R) Immunized mice
Percent Chromium Release
100
80
60
40
20
0
-20
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
B6
CBA
5R
E3
Tm211
P815

Fig.13

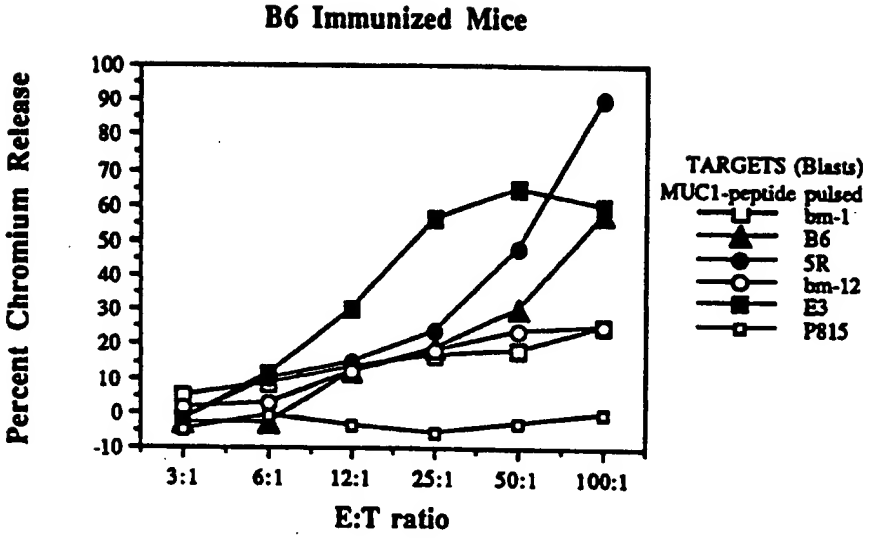

B6 Immunized Mice
Percent Chromium Release
100
90
80
70
60
50
40
30
20
10
0
-10
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
bm-1
B6
SR
bm-12
E3
P815

Fig.14

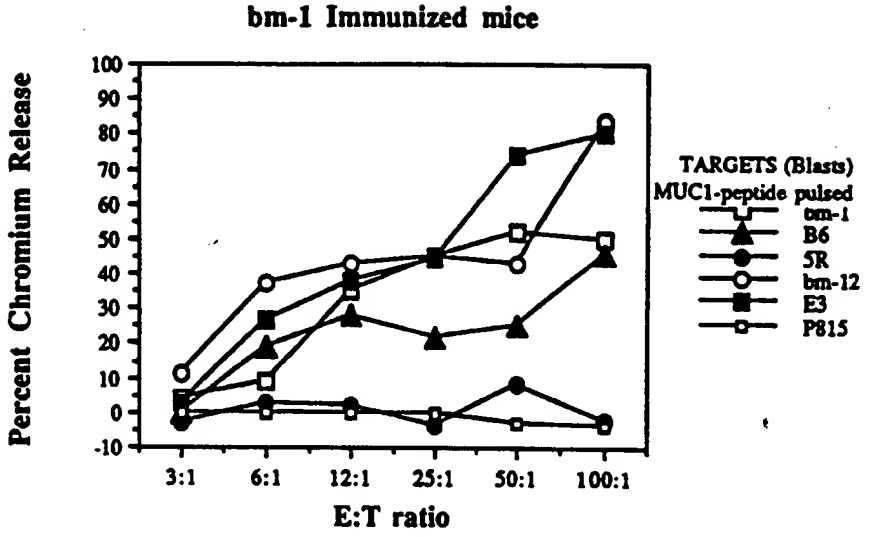

bm-1 Immunized mice
Percent Chromium Release
100
90
80
70
60
50
40
30
20
10
0
-10
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
bm-1
B6
5R
bm-12
E3
P815

Fig.15

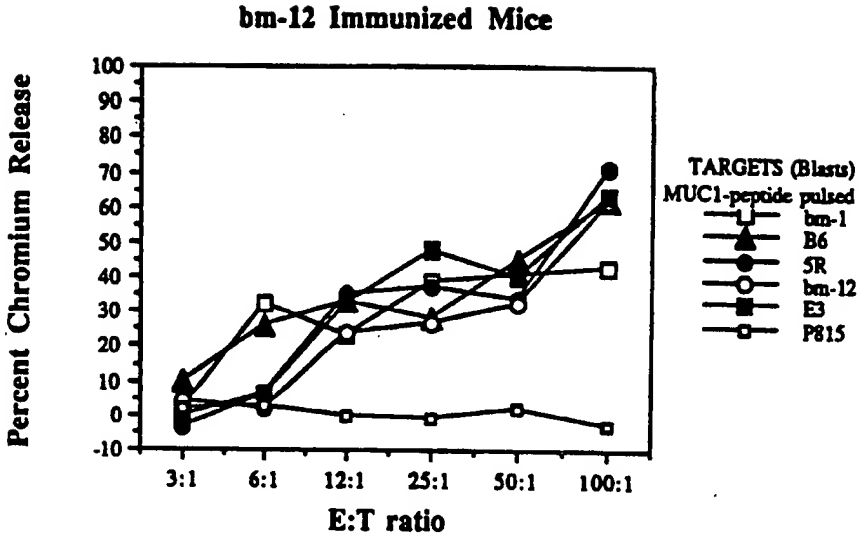

bm-12 Immunized Mice
Percent Chromium Release
100
90
80
70
60
50
40
30
20
10
0
-10
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
bm-1
B6
5R
bm-12
E3
P815

Fig.16

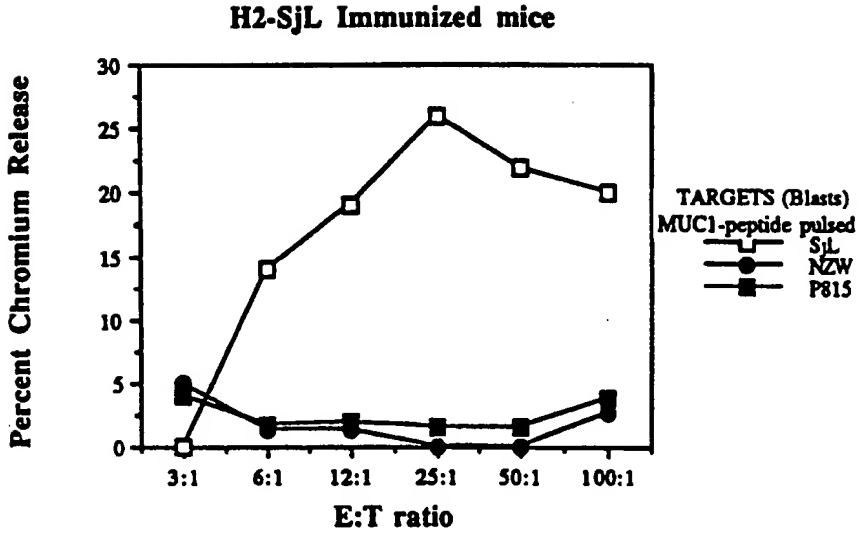

H2-SjL Immunized mice
Percent Chromium Release
30
25
20
15
10
5
0
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
SjL
NZW
P815

Fig.17

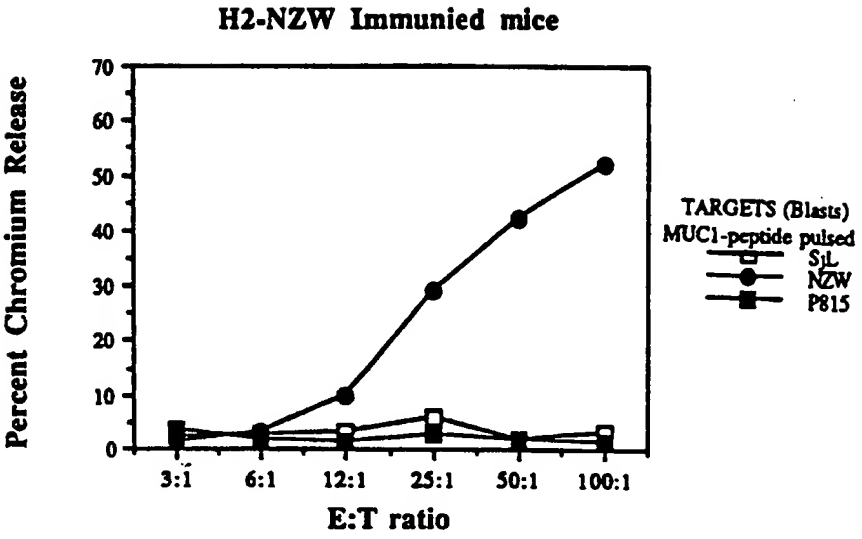

H2-NZW Immunied mice
Percent Chromium Release
70
60
50
40
30
20
10
0
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
SJL
NZW
P815

Fig.18

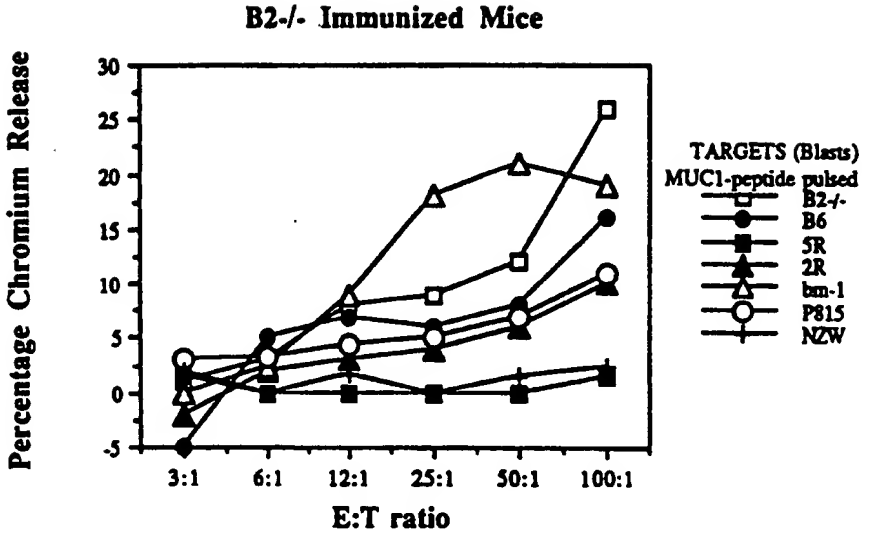
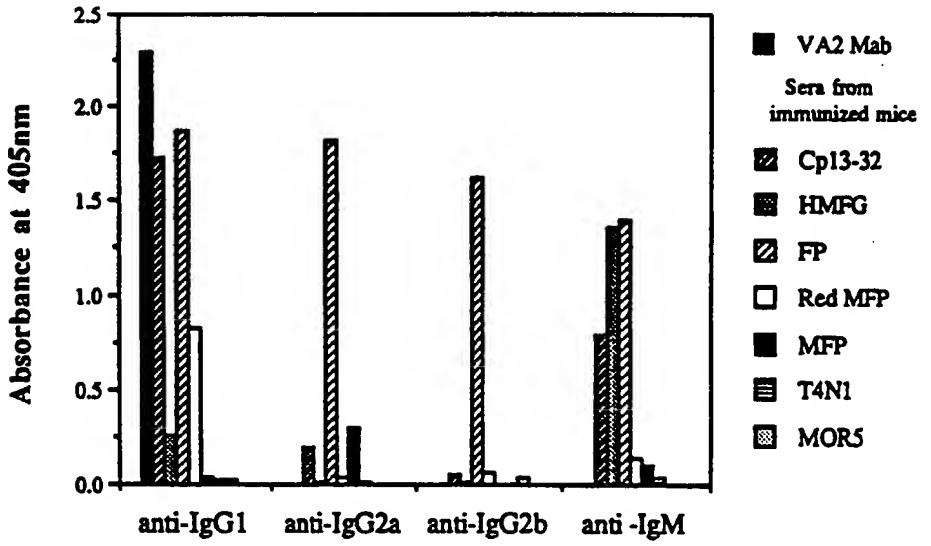

B2-/- Immunized Mice
Percentage Chromium Release
30
25
20
15
10
5
0
-5
3:1
6:1
12:1
25:1
50:1
100:1
E:T ratio
TARGETS (Blasts)
MUC1-peptide pulsed
B2-/-
B6
5R
2R
bm-1
P815
NZW

Fig.19

A


MUC1-Fusion Protein Coated Plate
Absorbance at 405nm
2.5
2.0
1.5
1.0
0.5
0.0
anti-IgG1
anti-IgG2a
anti-IgG2b
anti -IgM
VA2 Mab
Sera from immunized mice
Cp13-32
HMFG
FP
Red MFP
MFP
T4N1
MOR5

B

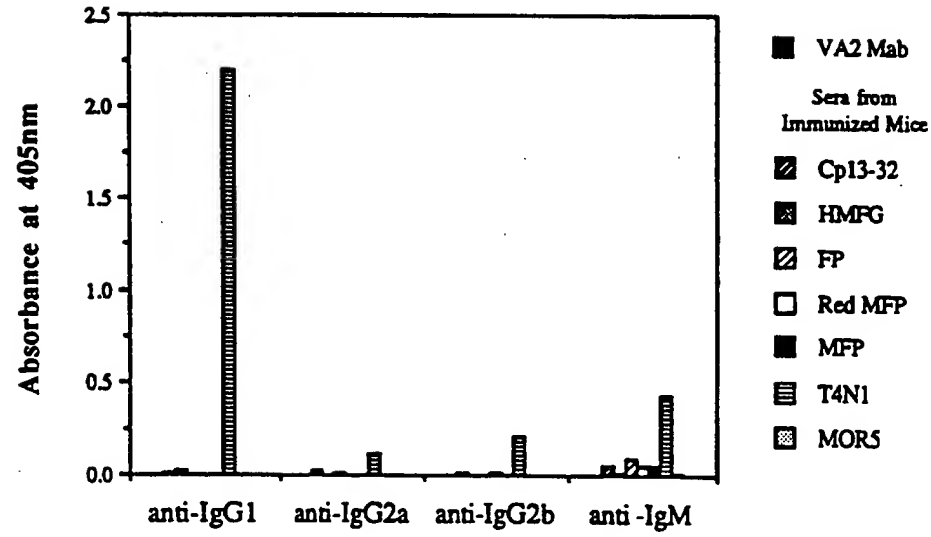

T4N1 Coated Plate
Absorbance at 405nm
2.5
2.0
1.5
1.0
0.5
0.0
anti-IgG1
anti-IgG2a
anti-IgG2b
anti -IgM
VA2 Mab
Sera from Immunized Mice
Cp13-32
HMFG
FP
Red MFP
MFP
T4N1
MOR5

Fig.20

COUPLING OF MUC 1 FUSION PROTEIN TO MANNAN

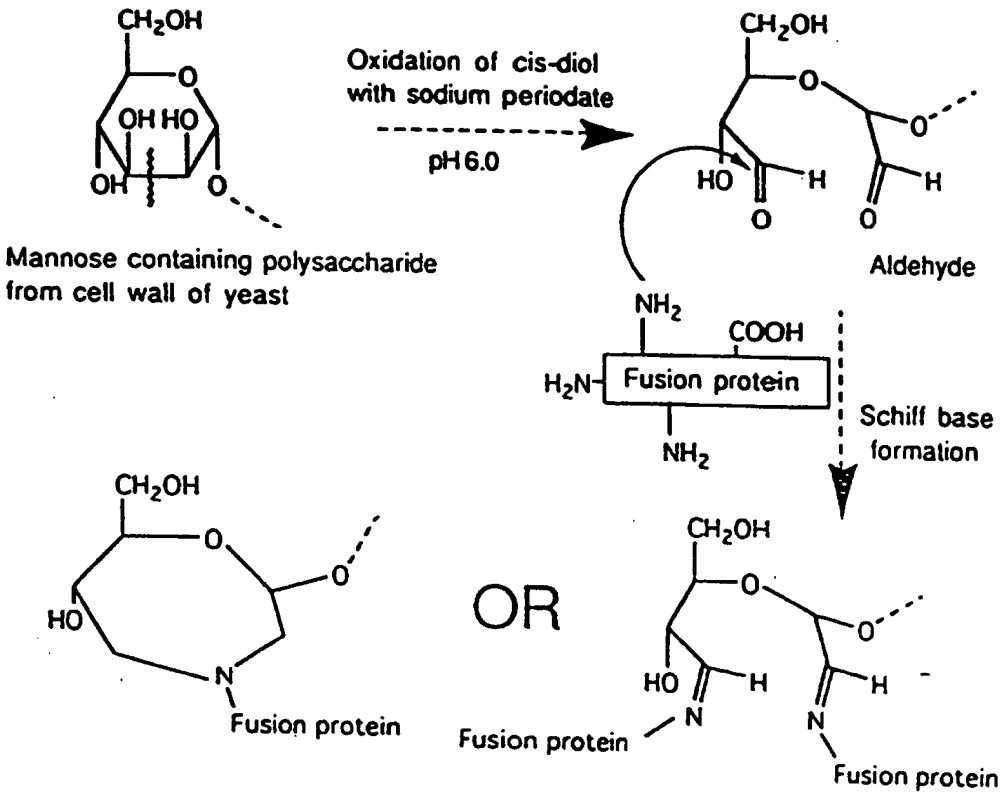


Figure 21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 94/00789

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.[6] C07K 9/00, 14/47, 14/705, 17/10, 19/00; A61K 39/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC: FILE WPAT: as below

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
AU: IPC: C07K 9/00, 13/00, 15/06

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
FILE WPAT: MUCIN, CONJUGATE, CONJUGATION, CARBOHYDRATE, SUGAR, POLYSACCHARIDE, OLIGOSACCHARIDE.
FILE CASM: MUCIN AND CONJUGATE, CONJUGATION

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	AU,B,41876/89 (620149) (THE AUSTRALIAN NATIONAL UNIVERSITY) 23 March 1990 (23.03.90) see page 2 lines 1 to 24, claim 5	1,6,7,13
X	AU,A,37462/93 (BIOCINE SCLAVO SPA) 5 October 1993 (05.10.93) see abstract	1,2,7,8,13,14
P,X	AU,A,56757/94 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 23 June 1994 (23.06.94) see abstract, page 7 line 15	1,2

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 March 1995 (27.03.95)	10 April 1995

Name and mailing address of the ISA/AU

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Telephone No. (06) 2832295

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	AU,A,34783/93 (SOLVAY ANIMAL HEALTH, INC) 3 August 1993 (03.08.93) see entire document, page 3 lines 21 to 33.	1 to 27
Y	WO, 89/08711, (THE BIOMEMBRANE INSTITUTE) 21 September 1989 (21.09.89) see entire document , pages 2, 7, 38, 66.	1 to 27
Y	G Denton, M Sekowski and M R Price, "Induction of antibody responses to breast carcinoma associated mucins using synthetic peptide constricts as immunogens" Cancer Letters, Vol 70 (1993) pages 143 to 150. see entire document, last line of summary.	1 to 27
Y	L Ding, E Lalani, M Reddish, R Kogarty, T Wong, J Samuel, M B Yacyshyn, A Meikle, P Ys Fung, J Taylor-Papadimitriou, B M Longenecker, "Immunogenicity of synthetic peptides related to the core peptide sequence encoded by the human MUCI mucin gene: effect of immunization on the growth of mucine mammary adenocarcinoma cells transfected with the human MUCI gene", Cancer Immunology Immunotherapy, Vol 36 (1993) pages 9 to 17. see entire document, last line of abstract.	1 to 27
Y	F Hudecz and M R Price "Monoclonal antibody binding to peptide epitopes conjugated to synthetic branched chain polypeptide carriers", Journal of Immunological Methods, Vol 147 (1992) pages 201 to 210. see entire document, last line of abstract.	1 to 27
Y	Jerome, Keith R et al, "Cytotoxic T-Lymphocytes Derived from Patients with Breast Adenocarcinoma Recognize and Epitope Present on the Protein Core of a Mucin Molecule Preferentially Expressed by Malignant Cells", Cancer Research 51, pp 2908-2916, (June 1 1991). see entire document	1 to 27
Y	Jerome, Keith R et al, "Expression of Tumor-associated Epitopes on Epstein-Barr Virus-immortalized B-cells and Burkitt's Lymphomas Transfected with Epithelial Mucin Complementary DNA", Cancer Research 52, 5985-5990, (November 1, 1992). see entire document	1 to 27
Y	Fontenot, Darrell, J, "Biophysical Characterization of One-, Two-, and Three-Tandem Repeats of Human Mucin (muc-1) Protein Core", Cancer Research 53, pp 5386-5394, (November 15, 1993). see abstract and introduction.	1 to 27
Y	Lalani, El-Nasir, et al, "Expression of the Gene Coding for a Human Mucin in Mouse Mammary Tumor Cells Can Affect Their Tumorigenicity", J. Biol. Chem. Vol 266, No. 23, pp 15420-15426, (August 15, 1991). see entire document	1 to 27
Y	Apostolopoulos, V; Xing, P X; Trapani, J A; McKenzie, I F C, "Production of anti-breast cancer monoclonal antibodies using a glutathione-S-transferase-MUCI-bacterial fusion protein", Br. J. Cancer (1993) 67, pp 713-720. see whole document.	1 to 27

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU	41876/89	EP	431023	JP	4501105	WO	9001949
AU	37462/93	EP EP	632727 566545	IT	92300058	WO	9317712
AU	56757/94	WO	9413312				
AU	34783/93	EP	626008	IL	104382	WO	9314195
WO	8908711	EP	357767	JP	2503387	US	5229289

END OF ANNEX